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Cell Wall Integrity Signaling in *Saccharomyces cerevisiae*

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INTRODUCTION

The Yeast Cell Wall

Yeasts are unicellular fungi which in the wild typically live on the surface of plants such as fruits and flowers. Existing in this setting, yeast cells face the potential for exposure to rapid and extreme changes in environment, particularly with respect to osmotic potential. For instance, a yeast cell living on the sugar-rich tissue of a grape can be exposed instantaneously to the hypo-osmotic shock of rainfall. To survive such rapid decreases in extracellular osmolarity, the cell must limit the influx of water so as to avoid bursting and to maintain an intracellular water activity that is appropriate for biochemical reactions (121, 313). Yeasts and other fungi have solved this problem with strong and relatively rigid cell walls that limit swelling. The fungal cell establishes a balance by which the force driving water across the osmotic gradient into the cell is counteracted by turgor pressure against the plasma membrane and cell wall.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is also required to maintain cell shape (50, 159), which is essential for the formation of a bud and hence cell division. The cell must remodel this rigid structure to accommodate cell expansion during vegetative proliferation, mating pheromone-induced morphogenesis, and nutrient-driven filamentation. Turgor pressure is critical for cell expansion, because it provides the force to overcome molecular cohesion within the cell wall (109). Because fungal cells maintain an intracellular osmolarity that exceeds that of the extracellular environment, water tends to flow into the cell, thereby providing turgor pressure. However, this pressure is equally distributed across the cell surface. Therefore, for growth to produce cell shapes other than spheres, cell wall expansion must be focused to particular regions. *Saccharomyces cerevisiae* uses an internal actin cytoskeleton for this purpose (77). During periods of polarized cell growth, the wall is loosened by digestive enzymes (e.g., glucanases and chitinases) and expanded at a single point on the cell surface. Wall remodeling must be carried out in a highly regulated manner—the growth site is loosened enough to allow expansion but not so much as to risk rupture.

Yeast cells invest considerable energy toward biogenesis of the cell wall, which comprises some 20 to 30% of the cell dry weight (243, 313). The major features of the *Saccharomyces cerevisiae* cell wall architecture are now fairly well understood. For a recent review on its molecular organization, the reader is referred to Klis et al. (160). Briefly, the cell wall is a layered structure with an electron-transparent inner layer and an electron-dense outer layer (40, 244). The inner layer is comprised of glucan polymers and chitin (*N*-acetylglucosamine polymers). This layer is constructed mainly (80 to 90%) of β1,3-glucan chains with some β1,6-linked glucan branches. Polymers of β1,6-glucan chains make up most of the remainder of the inner layer (8 to 18%), with chitin chains representing the smallest fraction (1 to 2%). This layer is largely responsible for the mechanical strength and elasticity of the cell wall owing primarily to the helical nature of β1,3-glucan chains (270, 313).

The outer cell wall layer is a lattice of highly glycosylated mannoproteins, which functions to protect the glucan layer from wall-degrading enzymes (68, 69, 160, 372). It is also important for cell-cell recognition during sexual agglutination and biofilm formation (40, 186, 273). Two major classes of cell surface glycoproteins comprise the outer cell wall layer. Members of one class, called glycosylphosphatidylinositol (GPI) proteins, are directed through the secretory pathway to the extracellular face of the plasma membrane by lipid anchors at their C termini. GPI-proteins are liberated from the plasma membrane by cleavage of their anchors prior to attachment to the cell wall (164). Among the approximately 70 GPI-proteins identified in the *Saccharomyces cerevisiae* genome (41), it is estimated that half reside in the cell wall (313). The other major class of cell wall proteins is represented by four related polypeptides, Pir1 to Pir4 (152, 228, 330). Although the Pir proteins appear to be linked directly to the β1,3-glucan-chitin lattice, GPI-proteins are generally linked to β1,3-glucan indirectly through a connecting β1,6-glucan chain (160).

Cell Wall as a Target for Antifungal Drug Development

A diverse group of fungi, including various *Candida* species, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, are the major opportunistic human pathogens causing systemic infections among immune-compromised patients (102). As the immune-compromised population has grown over the last three decades due to human immunodeficiency virus infection, cancer chemotherapy, and organ transplants, the number of life-threatening systemic fungal infections has increased accordingly (325). Effective antifungal therapy is currently very limited and dominated by the azole class of ergosterol biosynthesis inhibitors (90, 98). Members of this class of antifungals are cytostatic rather than cytotoxic and therefore require long therapeutic regimens. Consequently, there is a drive to develop safe, cytotoxic antifungal drugs. Because of the importance of the fungal cell wall for survival and morphogenesis and because human cells do not have walls, this structure is regarded as a prime target for the development of safe and effective antifungal agents (38, 99). *Saccharomyces cerevisiae* is considered a good model for the study of fungal cell wall biogenesis. Many aspects of cell wall construction and stress signaling are conserved between *Saccharomyces cerevisiae* and pathogenic species, most notably *Candida albicans*, another species of budding yeast.

The focus of this review is the regulatory pathways employed by *Saccharomyces cerevisiae* to maintain cell wall integrity during growth and morphogenesis and in the face of external challenges that cause cell wall stress. Although several signaling pathways contribute to the maintenance of the cell wall, the one principally responsible for orchestrating changes to the wall and responding to challenges to this structure is known as the cell wall integrity pathway, which will be abbreviated hereafter as the CWI pathway. I will also discuss recent advances in our understanding of how this pathway interfaces with other signaling pathways. In particular, several signaling pathways converge to regulate organization of the actin cytoskeleton. Some of these pathways also regulate CWI signaling, presumably to coordinate cell polarization with cell wall biogenesis. I will not discuss in depth the related topic of osmoregulation except as it relates to CWI signaling. An excellent recent review deals with adaptation to both hyper- and hypo-osmotic stress in yeast (121).

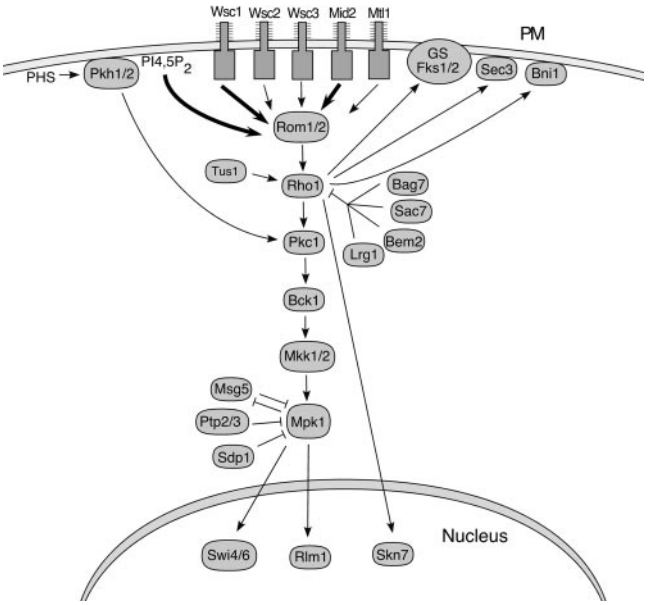

FIG. 1. CWI signaling pathway. Signals are initiated at the plasma membrane (PM) through the cell surface sensors Wsc1, -2, and -3, Mid2, and Mtl1. The extracellular domains of these proteins are highly O-mannosylated. Together with $PI4,5P_2$, which recruits the Rom1/2 GEFs to the plasma membrane, the sensors stimulate nucleotide exchange on Rho1. Relative input of each sensor is indicated by the width of the arrows. Rho1 activates five effectors, including the Pkc1-MAP kinase cascade, the β1,3-glucan synthase (GS), the Bni1 formin protein, the exocyst component Sec3, and the Skn7 transcription factor. Additional regulatory inputs from the Tus1 GEF, the inhibitory Rho1 GAPs, and the Pkh1/2 protein kinases are indicated. Pkh1/2 are activated by phytosphingosine (PHS). The MAP kinase cascade, which is comprised of Bck1, Mkk1/2, and Mpk1, is activated by Pkc1. Several MAP kinase phosphatases downregulate Mpk1. Two transcription factors, Rlm1 and the SBF complex (Swi4/Swi6), are targets of the MAP kinase.

CWI PATHWAY ARCHITECTURE

The CWI signaling pathway is comprised of a family of cell surface sensors coupled to a small G-protein called Rho1, which activates a set of effectors, each of which will be considered individually. Collectively, these effectors regulate a diverse set of processes including, but not limited to, β-glucan synthesis at the site of wall remodeling, gene expression related to cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site. A diagrammatic representation of the core elements of this pathway is presented in Fig. 1.

CWI MAP Kinase Cascade

The Rho1 effector pathway that has been studied in the greatest detail is the Pkc1-activated mitogen-activated protein (MAP) kinase cascade. This is principally because mutants in this pathway display striking but conditional cell lysis defects that render them genetically tractable. A linear series of protein kinases, known as a MAP kinase cascade, is responsible for amplification of the CWI signal from Rho1. The details of isolation and validation of the various components of the CWI MAP kinase cascade have been reviewed extensively (104, 114, 121, 119, 179). It is one of five MAP kinase signaling pathways in yeast that regulate mating, response to high osmolarity, pseudohyphal/invasive growth, sporulation, and response to cell wall stress.

Briefly, the MAP kinase cascade for CWI signaling is a linear pathway comprised of Pkc1 (180), an MEKK (Bck1) (55, 174), a pair of redundant MEKs (Mkk1/2) (137), and a MAP kinase (Mpk1/Slt2) (173, 204). A combination of genetic and biochemical studies have established that Pkc1 activates Bck1, which activates Mkk1/2, which in turn activate Mpk1. What is the purpose of such an arrangement? Studies by Ferrell and colleagues have demonstrated that MAP kinase cascades serve both to amplify a small signal initiated at the cell surface and to convert a graded input to a highly sensitive, switch-like response (84, 127). A cascade will not be triggered by signals of low magnitude or duration (noise) but will respond rapidly and fully to stimuli that reach their threshold for activation.

Pkc1 phosphorylates Bck1 in vitro at several sites (Ser939, Thr1119, and Ser1134) in a hinge region between its putative regulatory domain and its catalytic domain (178). Significantly, activating mutations in *BCK1* also cluster in this region of the protein (174). Of particular importance is the Thr1119 phosphorylation site, which is mutated to proline in the constitutive *BCK1-19* allele (174). Mutation of Thr1119 to Ala, Cys, or Tyr also results in constitutive signaling (D. E. Levin and A. K. Sobering, unpublished), suggesting that disruption of an interaction involving Thr1119 (either by phosphorylation or mutation) is the key to activation of this MEKK. Bck1 is presumed to phosphorylate and activate Mkk1/2 based on genetic epistasis studies, two-hybrid interaction, and its requirement for activation of Mpk1 (137, 148, 254). Mkk1/2 phosphorylate Mpk1 on neighboring tyrosyl and threonyl residues in a T-X-Y motif that is diagnostic for MAP kinases. This MAP kinase is conserved in *Candida albicans*, and is required for maintenance of cell wall integrity in that species (234).

Loss of function of any protein kinase below Pkc1 (or both Mkk1 and Mkk2) results in cell lysis at elevated growth temperature. The growth defects of these mutants are osmoremedial (e.g., with 1 M sorbitol), consistent with a primary defect in cell wall biogenesis. Loss of *PKC1* results in osmoremedial cell lysis at all growth temperatures (177, 253), prompting the suggestion that Pkc1 regulates additional targets that are separate from the MAP kinase cascade (174). Secondary Pkc1 targets are discussed in the section on alternative Pkc1 pathway branches. Other phenotypes associated with mutants in the CWI MAP kinase cascade include sensitivity to mating pheromone and cell wall antagonists such as calcofluor white, Congo red, caffeine, and the wall lytic enzyme zymolyase (81, 205), and actin polarization defects (212).

Mpk1 resides predominantly in the nucleus under nonstress conditions but rapidly relocates to the cytoplasm in response to cell wall stress (148). A small pool of Mpk1 localizes to sites of polarized cell growth and shuttles constitutively between these sites and the nucleus (341). During pheromone-induced morphogenesis, a minor pool of Mpk1 can be detected at the shmoo tip (18, 341). Bud tip and bud neck localization of Mpk1 evidently does not require the actin cytoskeleton because it is not disrupted by treatment with the actin antagonist latrunculin A, which interferes with actin polymerization (225). However, polarized localization of Mpk1 during growth and mor-

phogenesis does require Spa2, a component of the polarisome, which functions in actin cytoskeleton organization (197).

Mkk1 and Mkk2 are mainly cytoplasmic proteins, but, like Mpk1, they can be detected at sites of polarized growth in an Spa2-dependent manner. Moreover, Spa2 displays two-hybrid interactions with both Mpk1 and Mkk1/2 (341), leading to the suggestion that Spa2 serves as a scaffold for these protein kinases. However, in contrast to the role of the Ste5 scaffold protein in activation of the pheromone response MAP kinase cascade (79), Spa2 is not required for Mpk1 activation during vegetative growth or in response to pheromone treatment (35, 304). This finding suggests that the function of the Spa2 scaffold with regard to CWI signaling is to focus the action of the kinases to the site of polarized growth. Interestingly, Bck1 has been detected in the cytoplasm but not at polarized growth sites (341). However, Bck1 is expressed at low abundance compared with Mpk1 and Mkk1/2 (D. E. Levin, unpublished), leaving open the possibility that a small pool of polarized Bck1 has escaped detection.

Rho GTPases: Rho1-5 and Cdc42

The RHO family of GTPases play a central role in polarized growth in animal and fungal cells alike (77, 275). *Saccharomyces cerevisiae* possesses six Rho-type GTPases, named Rho1 to Rho5 and Cdc42. They reside at the plasma membrane and serve related but distinct roles in cell polarity establishment and maintenance. Cdc42 function is important for bud site assembly and is essential for the establishment of polarized growth (141, 142). It also functions in a late step in exocytosis (1). Rho1 is an essential protein that controls CWI signaling and will be discussed in more detail below. It is important for protein kinase C (Pkc1) activation, β1,3-glucan synthase (GS) activity, and actin cytoskeleton organization, and plays a role in polarized secretion (reviewed in reference 37). Rho2 is nonessential and partially redundant with Rho1 (116, 195, 246). Rho3 is nearly essential for cell growth—conditional mutants display cell polarity and lysis defects (208). It has been suggested that Rho3 shares a role in actin polarization and bud formation with the nonessential Rho4 (132, 145, 208). Rho3 appears to have additional functions at two steps in the exocytic pathway, which may not be shared with Rho4 (1).

Rho5 was the last family member to be discovered (283), but the absence of a phenotypic defect associated with its deletion has left its function enigmatic. A recent report suggests that it functions to downregulate the CWI pathway (295). This was based on the finding that a *rho5*Δ mutant displays elevated basal and stress-induced Mpk1 activity and increased resistance to cell wall stressors. However, if Rho5, like the other Rho family members, serves a function in cell polarity that is separate from CWI signaling, the effect of its loss of function on Mpk1 activity may be indirect.

Rho proteins are C-terminally prenylated, a modification that increases their hydrophobicity and allows their association with membranes. These modifications are essential for their proper localization and function (288). The essential GTPases, Rho1 and Cdc42, are modified by the action of the Cdc43/Ram2 geranylgeranyl transferase (135). The absence of prenylation renders Rho1 soluble and unable to activate or even interact with GS (135). For this reason, geranylgeranyl transferase 1 is regarded as a potential target for the development of antifungal drugs (154). Inspection of their C-terminal sequences suggests that Rho2 and Rho5 are likely also modified by geranylgeranyl transferase 1, whereas Rho3 and Rho4 are probably substrates for the Ram1/Ram2 farnesyl transferase.

Rho1: Master Regulator of CWI Signaling.

Rho1 is considered the master regulator of CWI signaling not only because it receives the major inputs from the cell surface but also because it regulates a variety of outputs involved in cell wall biogenesis, actin organization, and polarized secretion (Fig. 1). Moreover, it seems likely that Rho1 coordinates these functions at the cell surface. It is localized to sites of polarized growth in a manner dependent on the actin cytoskeleton (16, 265, 359).

Regulators of Rho1. Like other G-proteins, Rho1, the yeast homolog of mammalian RhoA (264), cycles between the active GTP-bound state and the inactive GDP-bound state. The Rho1 cycle is regulated both by GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) acting in opposition. Among the 11 Rho-GAPs identified in *Saccharomyces cerevisiae*, four have been shown to act on Rho1 both in vitro and in vivo—Bem2, Sac7, Bag7, and Lrg1 (49, 205, 256, 283, 292, 294, 347). Interestingly, these GAPs appear to regulate Rho in a target-specific manner. For example, Lrg1 is evidently dedicated to regulation of GS (347). Similarly, Bem2 and Sac7 are the only GAPs that regulate the Pkc1 MAP kinase pathway (205, 294). Bag7 and Sac7, which are most similar to each other rather than to any of the other GAPs, collaborate to control the actin cytoskeleton (292, 294). The apparently independent regulation of different Rho1-effector pairs by distinct GAPs indicates some compartmentalization of Rho1 functions. Different effectors may be active through the cell cycle or in response to different types of cell wall stress.

Rho1 is stimulated primarily through the action of the Rom1 and Rom2 GEFs (246). These GEFs provide a redundant function in the activation of Rho1 (and likely Rho2). Loss of *ROM2* function results in temperature-sensitive growth, whereas loss of both *ROM1* and *ROM2* is lethal. Like Rho1, Rom2 (and probably Rom1) resides at sites of polarized growth (201). Rom1 and Rom2 have Dbl homology (DH) domains, which interact with GDP-bound Rho1 and possess the nucleotide exchange activity of these proteins (246). They also possess pleckstrin homology (PH) domains, which bind phosphatidylinositol-4,5-biphosphate ($PI4,5P_2$) and are responsible for proper localization of Rom1/2 to the plasma membrane (11).

An N-terminal domain of Rom2 (and presumably Rom1) that is separate from either the DH or PH domains is responsible for associating with Wsc1 and Mid2 and likely other cell surface sensors (257). A third Rho1-GEF, Tus1, was isolated recently as a dosage suppressor of the growth and cell lysis defect of a *ypk1/2* mutant (291). A *tus1*Δ mutant displays an osmoremedial growth defect at high temperature, which is also suppressed by overexpression of CWI pathway components (i.e., Rom2, Rho1, or Rho2). Tus1 possesses both a DH domain that interacts with GDP-bound Rho1 and an uncharacterized PH domain (291). Study of its PH domain has thus far failed to establish a role in phospholipid binding (367). Functional distinctions between Tus1 and Rom1/2 have not yet

been made, nor is it clear if Tus1 responds to the cell surface sensor proteins in the same way as its cousins. Clarification of the role of Tus1 in CWI signaling is needed.

Saccharomyces cerevisiae possesses a single Rho-GDP dissociation inhibitor (RhoGDI), designated Rdi1, which associates with both Rho1 and Cdc42 (162, 206). Mammalian RhoGDIs stimulate release of Rho GTPases from membranes (123, 238, 357) and block GDP dissociation and GTP hydrolysis by interfering with the actions of GEFs and GAPs (113). Deletion of *Saccharomyces cerevisiae RDI1* results in no detectable phenotypic defects (206), but its overexpression inhibits growth and induces increased steady-state levels of Rho1 and Cdc42, consistent with a role in the inhibition of these G-proteins (162). Rdi1 was shown recently to localize to sites of polarized growth, and its overexpression caused delocalization of Cdc42 from membranes to the cytoplasm (274). Rdi1 may facilitate the relocalization of Rho1 and Cdc42 from the bud tip to the mother/bud neck, but this possibility has not been addressed directly.

Targets of Rho1. Five effectors for Rho1 have been described—the Pkc1 protein kinase, the β1,3-glucan synthase (GS), the Bni1 and Bnr1 formin proteins, the Skn7 transcription factor, and the Sec3 exocyst component. As indicated above, evidence is accumulating to suggest that each Rho1-effector pair is regulated separately by a different complement of GAPs and perhaps different GEFs as well. Together, these effectors regulate synthesis of cell wall glucans and chitin, expression of genes important for cell wall biogenesis, polarization of the actin cytoskeleton, and perhaps exocytosis.

Pkc1. Mammalian cells possess at least 10 isoforms of protein kinase C (PKC) and two additional PKC-related kinases (215). By contrast, the *Saccharomyces cerevisiae* genome encodes only a single homolog of mammalian protein kinase C, designated Pkc1 (180). It was the first component of the CWI signaling pathway discovered, and although this protein kinase likely has several intracellular substrates, only its regulation of the Bck1-Mkk1/2-Mpk1 MAP kinase cascade has been well studied. Deletion of *PKC1* is lethal under normal growth conditions, but the viability of a *pkc1*Δ mutant can be rescued by osmotic support (177, 180, 253). Loss of *PKC1* results in a more severe growth defect than that displayed by deletion of any of the members of the MAP kinase cascade under the control of Pkc1, which prompted the suggestion that Pkc1 regulates multiple pathways (174). Pkc1 was recently identified as the target of cercosporamide, a potent natural antifungal product whose antifungal activity was widely recognized but whose mechanism of action was not understood (325).

Electron micrographic images of *pkc1*Δ cells maintained in the presence of osmotic support suggest a pleiotropic set of cell wall defects (178, 281). Both the inner, glucan-containing layer and the outer, mannoprotein layer are thinner in *pkc1*Δ mutants. These alterations are mirrored in a reduction in both β1,3- and β1,6-glucans of approximately 30% and a reduction in mannan of approximately 20% (281, 306). Additionally, the plasma membrane of *pkc1*Δ mutants appears to separate from the cell wall at some points (178, 253).

Pkc1 associates with and is activated by GTP-bound Rho1 (149, 240), which confers upon the protein kinase the ability to be stimulated by phosphatidylserine (PS) as a lone cofactor (149). Cofactors that activate conventional PKCs, such as diacylglycerol and Ca^{2+}, do not regulate Pkc1 (9, 348). Consistent with this finding, a *pkc1*Δ mutant is complemented by human PKC-eta (239), a member of the novel subfamily of PKCs which do not respond to diacylglycerol or Ca^{2+}. PKC isoforms from other fungal species studied to date share with Pkc1 the requirements for Rho and phosphatidylserine (176, 325).

Pkc1 is larger than any of the mammalian PKCs owing to an extended regulatory domain that possesses all of the subdomains that are differentially distributed among various PKC and PKC-related kinase isoforms. It possesses two homologous region (HR1) domains (HR1A and B) at its N terminus (215), which are found in proteins that bind to RhoA, including the PKC-related kinases (305). Indeed, HR1 domains have been shown to be sites of RhoA interaction (88, 305), and the HR1A domain of Pkc1 contributes to the interaction of this kinase with Rho1 (297).

Pkc1 possesses a cysteine-rich domain, also known as a C1 domain, which is defined by a pair of zinc finger motifs. The C1 domain in the conventional mammalian PKCs is the site of diacylglycerol and phorbol ester binding (146, 326). However, despite the apparent importance of these putative zinc fingers to the function of Pkc1 (140), biochemical evidence indicates that this enzyme is not responsive to either diacylglycerol or phorbol ester (9, 348), even in the presence of GTP-bound Rho1 (149). Interestingly, the Pkc1 C1 domain lacks several residues that are essential for diacylglycerol/phorbol ester binding (340). Rather, the C1 domain of Pkc1 appears to be a second site for interaction with Rho1 (240, 296).

The C2 domain, also found in the conventional PKCs, is responsible for binding phospholipids in a Ca^{2+}-dependent manner (24). This domain is characterized by a set of five conserved aspartate residues that coordinate two Ca^{2+} ions (302). The Ca^{2+}-independent PKC isoforms possess C2-like domains that lack one or more of the conserved aspartate residues. Imperfect C2 domains may be responsible for the Ca^{2+}-independent phospholipid activation displayed by these isoforms, but this has not been demonstrated directly. Pkc1 possesses two imperfect C2 domains that flank the C1 domain (180, 215). Thus, activation of Pkc1 by phosphatidylserine/Rho1 likely involves the C2 domain (for phosphatidylserine binding) and the HR1 domain and the C1 domain (for Rho1 binding). Some C2 and C2-like domains form specific protein-protein interactions. For example, receptors for activated PKCs bind to the C2 domains of their cognate protein kinases and seem to be important for targeting them to specific membrane compartments (219). In this regard, it is noteworthy that a two-hybrid fusion containing the C1 and C2 domains of Pkc1 interacts with the endoplasmic reticulum luminal domains of several subunits of the oligosaccharide transferase complex (255). This raises the intriguing possibility that some Pkc1 may reside within the lumen of the endoplasmic reticulum to regulate protein glycosylation.

Finally, all PKCs possess a pseudosubstrate site, typically positioned immediately N-terminal to the C1 domain (236). A pseudosubstrate site resembles a PKC phosphorylation site except that it has an alanine at the position that would be the phosphorylation target serine or threonine. Under conditions in which the PKC is not active, the pseudosubstrate site inhibits protein kinase activity through an intramolecular interaction

with the active site. Mutational incapacitation of the pseudosubstrate site results in cofactor-independent protein kinase activity. Pkc1 possesses a pseudosubstrate site which, when mutated, yields a constitutive form (348) that can suppress the growth defects of conditional *rho1* mutants (240).

An intracellular localization study of Pkc1 revealed that it resides at sites of polarized cell growth (8). Specifically, early in the cell cycle, Pkc1 was detected at the prebud site and at bud tips, a pattern that is very similar to that of Rho1 (265, 359). Later in the cell cycle, it becomes delocalized and finally relocalized at the mother-bud neck. The neck localization of Pkc1 requires an intact septin ring (64). A recent molecular dissection of Pkc1 suggested that each domain was responsible for localizing a pool of Pkc1 to various subcellular sites (64). For example, the HR1 domains are responsible for targeting Pkc1 to the bud tip and neck. This is consistent with the role these domains play in the association of Pkc1 with Rho1. The C1 domain in isolation localized to the cell periphery but in an unfocused manner, perhaps reflecting association of this domain with membrane lipids.

Surprisingly, removal of the HR1 domains resulted in localization of Pkc1 to the mitotic spindle, evidently directed by the N-terminal C2-like domain. Additionally, the C-terminal C2-like domain of Pkc1 (referred to by these authors as the interdomain) in isolation localized specifically to the nucleus, owing to a pair of nuclear import signals present within this region. A nuclear export signal residing within the HR1A domain is apparently responsible for maintaining the pool of nuclear Pkc1 at a low level. The existence of a nuclear pool of Pkc1 may ultimately help to explain some of the functions of this protein that are not connected to cell wall integrity (see below). Interestingly, there are several examples of metazoan PKCs colocalizing with microtubules (78, 175, 231, 345). Perhaps yeast Pkc1 is a better model for understanding some functions of higher eukaryotic PKCs than was previously believed.

Glucan synthase. Vegetative proliferation requires remodeling of the cell wall to accommodate its expansion at the growth site. The main structural components responsible for the rigidity of the yeast cell wall are β1,3-linked glucan polymers with some branches through β1,6 linkages (160). The biochemistry of the enzyme complex that catalyzes the synthesis of β1,3-glucan chains (β1,3-glucan synthase [GS]) has been studied extensively, and three components of this complex have been identified. The echinocandin antifungal agents, which interfere with the production of β1,3-glucans and are presumed to target the GS, constitute the leading class of experimental drugs directed at treating life-threatening fungal infections (352). A pair of closely related genes, *FKS1* and *FKS2* (for *FK*506-sensitive), encode alternative catalytic subunits of the GS (75, 136, 211, 268). Fks1 and Fks2 are large, multispanning integral membrane proteins, either one of which is sufficient for GS activity and cell viability. Unlike loss of Pkc1, however, loss of Fks1/2 is not suppressed by increased osmotic support. This is presumably because cell wall biosynthesis is completely shut down in an *fks1*Δ *fks2*Δ mutant. A third gene encoding a homolog of Fks1/2, called Fks3, has not been characterized. Rho1 is an essential regulatory subunit of the complex, serving to stimulate GS activity in a GTP-dependent manner (76, 210, 265). Both Rho1 and Fks1 localize to the plasma membrane at sites of cell wall remodeling—the bud tip during bud growth, and the bud neck during cytokinesis (265, 359). A more detailed localization study revealed that GS colocalizes with cortical actin patches and moves on the cell surface in a manner dependent on actin patch mobility (338). The GS is thought to extrude glucan chains from the plasma membrane for incorporation into the wall.

An elegant intragenic complementation analysis of a dozen conditional *rho1* alleles revealed that two of its essential functions could be separated (287). Mutants in one group were defective in GS activity, and mutants in the other group were defective in activating Pkc1. Mutants in one group could complement mutants in the other group. Accordingly, mutants specifically deficient in Pkc1 signaling display cell lysis defects at the restrictive temperature, whereas mutants deficient in GS activity arrested growth without cell lysis. Although some of the *rho1* mutations disrupted its physical interaction with the effector of diminished function, others did not. In the latter case, an inability of the mutant Rho1 to induce a conformational change in the effector is suggested.

The *FKS1* and *FKS2* genes differ primarily in the manner in which their expression is controlled. Under optimal growth conditions, *FKS1* is the predominantly expressed gene, and its mRNA levels fluctuate periodically through the cell cycle, peaking in late G_1 (130, 287). Cell cycle-regulated expression of *FKS1* is controlled by the SBF and MBF transcription factors but primarily by SBF (136, 211, 268, 318), which is comprised of Swi4 and Swi6 (7). Expression of *FKS1* is also weakly regulated by CWI signaling (130, 144) through the Mpk1-activated transcription factor Rlm1 (144).

Expression of *FKS2* is low under optimal growth conditions, but its expression is induced in response to treatment with mating pheromone, elevated growth temperature, high extracellular [Ca^{2+}], growth on poor carbon sources, entry into stationary phase, or in the absence of *FKS1* function (211, 371). The pathway for induction of *FKS2* expression by pheromone, $CaCl_2$, or loss of *FKS1* requires the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (96, 211), the target of FK506 action (89, 187). Because *FKS1* and *FKS2* provide a redundant but essential function, regulation of *FKS2* expression by calcineurin explains the sensitivity of *fks1* mutants to FK506 and their synthetic lethality with calcineurin mutants (96).

Overexpression of Mkk1 or the Mpk1-activated transcription factor Rlm1 suppresses the synthetic lethality of an *fks1*Δ *cnb1*Δ mutant (371). Moreover, expression of a constitutive form of calcineurin suppresses the growth defects of *pkc1*Δ and *mpk1*Δ mutants (96). This connection to CWI signaling was explained in part by the finding that calcineurin and the CWI pathway function in parallel to regulate *FKS2* expression, through separable promoter elements, under conditions of cell wall stress (371) (Fig. 2). In response to elevated growth temperature, the immediate transcriptional induction of *FKS2* is mediated by the calcineurin-activated transcription factor Crz1/Tcn1, which binds to a calcineurin-dependent response element (CDRE) within the *FKS2* promoter (320, 371). The core consensus binding sequence for Crz1 is a 6-bp motif (GAGGCTG) (366). Maintenance of high levels of *FKS2* expression under chronic stress is driven by the CWI pathway but only partially through Rlm1 (144, 371). The sustained tran-

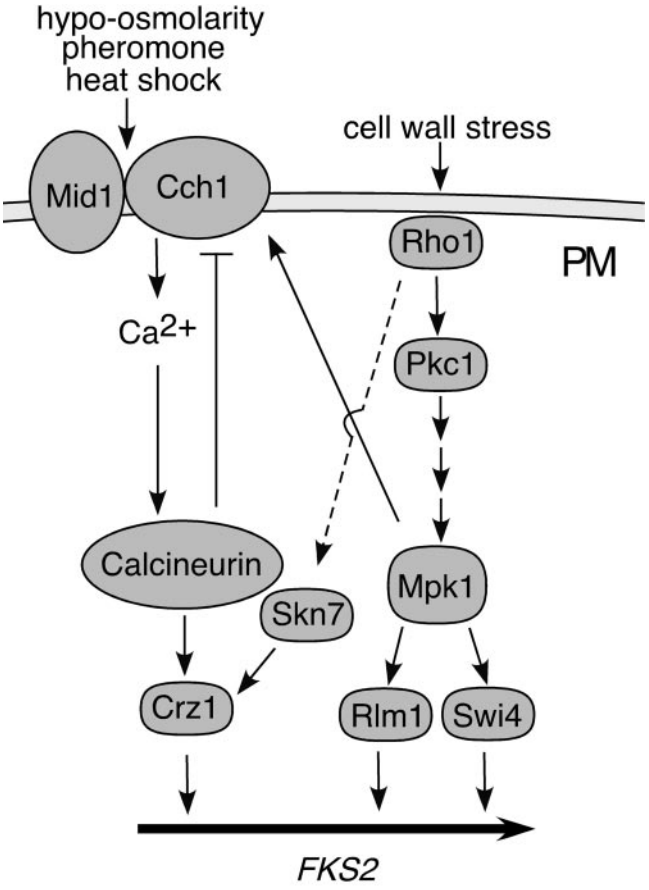

FIG. 2. Coordinate activation of CWI signaling, Ca^{2+} signaling, and Skn7 to induce *FKS2* expression. Activation of Mpk1 results in stimulation of Rlm1 and Swi4 transcription factors to drive transcription of *FKS2* (and other cell wall-related genes) and the Mid1-Cch1 Ca^{2+} channel. Channel activation leads to stimulation of the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin. Dephosphorylation of the Crz1 transcription factor by calcineurin allows its entry into the nucleus. Rho1 may activate the Skn7 transcription factor (hatched line), which drives both expression of the *OCH1* gene and stabilization of the Crz1 transcription factor for induction of *FKS2*. Thus, CWI signaling and Ca^{2+} signaling coordinately regulate expression of the GS-encoding *FKS2* gene at several levels.

scription of *FKS2* in response to wall stress appears to be regulated largely by Swi4 (D. E. Levin, K. Y. Kim, and U. S. Jung, unpublished), a second transcription factor that is activated by Mpk1. Therefore, Rho1 controls both the activity of the GS during normal growth and expression of its catalytic subunit under conditions of wall stress. Recent developments also implicate Rho1 at multiple levels in the activation of Crz1 (see Skn7 and Cch1/Mid1). The complex regulatory network centered on the induced expression of *FKS2* is evidently a mechanism to augment Fks1-derived GS activity under emergent conditions.

The site of β1,6-glucan synthesis has been controversial for many years. Genetic analyses revealed β1,6-glucan synthesis mutations in genes that function throughout the secretory pathway (249, 301), suggesting that biosynthesis of this polymer begins in the endoplasmic reticulum, progresses in the Golgi, and is completed at the cell surface. Indeed, a pair of functionally redundant glucosyl hydrolases (or transglucosylases) that are critical for β1,6-glucan synthesis, Kre6 and Skn1, reside in the Golgi (281). However, a late secretory pathway mutant displayed only surface labeling of the polymer, indicating that a secretory block does not result in accumulation of intracellular β1,6-glucan (221). This suggested that β1,6-glucan may, like β1,3-glucan, be synthesized at the plasma membrane.

A recent study describing an in vitro assay for β1,6-glucan synthesis revealed a requirement for GTP and, provocatively, demonstrated enhanced activity in cells overexpressing Rho1 (344). Thus, Rho1 may control the biosynthesis of both β-glucan polymers. If this is correct, it seems likely that β1,6-glucan synthesis is carried out at sites of polarized cell growth based on the localization pattern of Rho1. A further connection between β1,6-glucan synthesis and CWI signaling is the observation that a *pkc1*Δ mutant is suppressed by overexpression of *KRE6* (281). However, this may also be explained in terms of compensation for one wall defect by fortification of the glucan/chitin layer through an unrelated mechanism.

Bni1 and Bnr1. Formins nucleate the assembly of linear actin filaments in response to activation by Rho-GTPases. In yeast, the assembly of actin cables, which extend along the axis of growth and function as tracks for the transport of secretory vesicles into the bud, requires the functionally redundant formin proteins Bni1 (*B*ud *n*eck *i*nvolved) and Bnr1 (*Bn*i1 *r*elated) (83, 285, 286). Bni1 and Bnr1 are components of the polarisome that reside at the cell cortex during bud growth (247) and nucleate the assembly of actin cables emanating from the bud tip. Both proteins relocalize to the bud neck during cytokinesis (247), where they are essential for the formation of the actomyosin-based contractile ring (332).

Bni1 and Bnr1 are large, multidomain proteins that have been shown to associate with multiple Rho proteins through an N-terminal Rho-binding domain (RBD). The RBD negatively regulates formin activity by binding to a C-terminal autoregulatory domain (termed DAD) (3, 252). Association of GTP-bound Rho proteins to the RBD relieves the autoinhibitory interaction. Bni1 also interacts in two-hybrid and coprecipitation assays with actin (Act1) and three actin-binding proteins—profilin (Pfy1), Bud6, and translation elongation factor 1α (Tef1/2) (82, 133, 337). All of these actin-binding proteins have been implicated in the assembly or organization of actin filaments. Additionally, Bni1 associates directly with the cell polarity protein Spa2 (94), which is an early marker for sites of wall expansion (314).

Each Bni1-interacting component appears to have a separate binding site on the protein. Profilin binds to Bni1 through a proline-rich formin homology domain 1 (FH1), which is shared with Bnr1 and metazoan formins. A second domain that is highly conserved among family members, called FH2, nucleates actin polymerization in vitro (263, 285, 286). Tef1 and -2 bind in a region between the FH1 and FH2 domains, Bud6 associates with the C terminus, and the Spa2-binding site is located at midprotein. Thus, the emerging picture is one in which the formins serve as a platform for the assembly of an actin filament-producing machine (44).

The GTP-bound forms of Rho1, Rho3, Rho4, and Cdc42, bind to the RBD of Bni1 (82, 94, 163). Additionally, Bnr1 has been reported to interact with Rho4 (133). Recent genetic analyses, summarized below, have provided some insight into the function of various Rho proteins with respect to Bni1 and Bnr1. Rho3 and Rho4 have been known for some time to serve

a redundant role in bud growth that involves actin polarization (132, 208). As noted above, a *rho3*Δ mutant grows very slowly at all temperatures, and loss of *RHO4* function exacerbates this growth defect, resulting in cell lysis during bud expansion. Overexpression of Cdc42 but not Rho1 or Rho2 suppresses the growth and actin polarity defect of a *rho3*Δ *rho4*Δ mutant, suggesting some functional overlap (208).

It now appears that the essential function of Rho3 and Rho4 is to activate Bni1 and Bnr1 during bud growth, because constitutively activated forms of Bni1 (Bni1ΔRBD) or Bnr1 (Bnr1ΔRBD) suppress the growth defect of a *rho3*Δ *rho4*Δ mutant (74). By contrast, Rho1 is required for Bni1/Bnr1-mediated actin ring assembly during cytokinesis (332). A *rho1-2* mutant failed to form an actin ring at the mother-bud neck after release from nocodazole-induced M-phase arrest. Although neither *rho3* nor *rho4* mutants were examined in this study, a *cdc42-1* mutant was competent for actin ring formation. Thus, it seems that Rho1 drives actin polarization through Pkc1 and the MAP kinase cascade during bud growth and through Bni1/Bnr1 during cytokinesis.

Skn7. The HOG (*H*igh *O*smolarity *G*lycerol) pathway is controlled by a phosphorelay switch comprised of a cell surface sensor kinase (Sln1), a histidine phosphotransfer protein (Ypd1), and two response regulators (Skn7 and Ssk1) (155, 183, 199, 245, 261, 262). The Skn7 protein is one of only two yeast proteins related to bacterial response regulators of so-called two-component signal transduction pathways (183). Like many bacterial response regulators, Skn7 is a transcription factor. The other yeast response regulator, Ssk1, activates the MAP kinase cascade of the HOG (High Osmolarity Glycerol response) pathway. Whereas Ssk1 appears to be entirely under the control of Sln1, the lone sensor kinase of yeast, Skn7 activity is only partially regulated by Sln1. Skn7 may also be activated by Rho1 in response to cell wall stress (4) and through an unknown pathway in response to oxidative stress (167). Sln1 is a sensor of turgor pressure which is inactivated under conditions of low turgor (272). Sln1 regulates the HOG signaling pathway by phosphorylating the Ypd1 phosphorelay molecule, which transfers its phosphate to aspartyl residues within the receiver domains of both Ssk1 and Skn7, which constitute two branches of the same pathway (Fig. 3). Under high-osmolarity conditions, inactive Sln1 accumulates, resulting in dephospho-Ssk1, which is the active form of this response regulator. Thus, high extracellular osmolarity stimulates the Hog1 MAP kinase, which mediates, among other things, the biosynthesis and retention of glycerol as a compatible intracellular solute (5, 194, 328).

By contrast with Ssk1, dephospho-Skn7 is inactive. In response to hypo-osmotic stress (high turgor pressure), Sln1 activity inhibits Ssk1 and stimulates Skn7 by phosphorylation at Asp427. Thus, depending on the direction of the change in osmotic conditions, one or the other branch of the HOG pathway is activated. Hypo-osmotic activation of Skn7 results in the transcriptional activation of at least one gene, *OCH1* (184), which encodes a mannosyltransferase involved in maturation of N-glycoproteins, many of which are destined for the cell wall. Indeed, an *och1*Δ mutant is temperature sensitive, arresting growth at an early point in bud formation, and loses viability rapidly under restrictive conditions (229). This phenotype is reminiscent of conditional *pkc1* mutants and likely reflects cell lysis at the restrictive temperature. Phospho-Skn7 is also presumed to activate transcription of other genes involved in the response to hypo-osmotic stress.

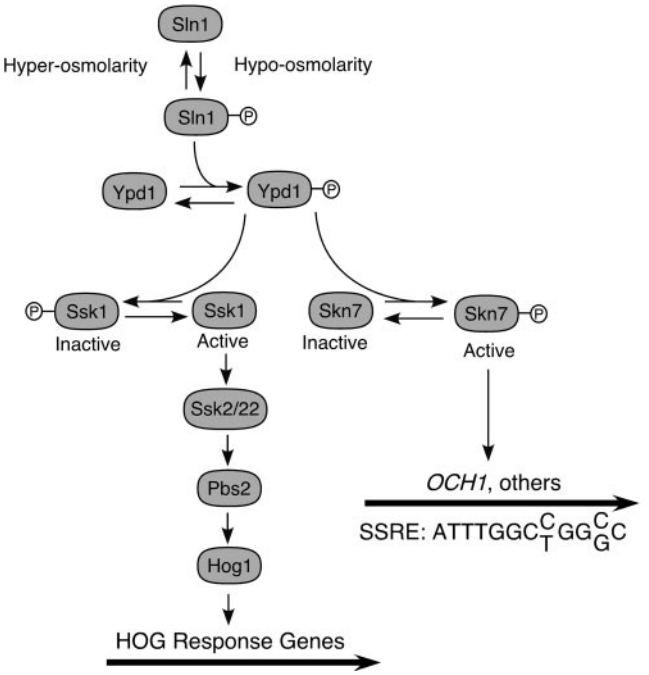

FIG. 3. Sln1 branch of the HOG pathway. The Sln1 sensor kinase is autophosphorylated under conditions of low extracellular osmolarity. Sln1 phosphorylates the histidine phosphotransfer protein Ypd1, which phosphorylates both the Skn7 and Ssk1 response regulators. Phospho-Skn7 is the active form of this transcription factor, which binds to the SSRE of *OCH1* and probably other cell wall-related genes. Phospho-Ssk1 is the inactive form of this response regulator. Sln1 is inactivated in response to high extracellular osmolarity. This allows the dephosphorylated forms of Ypd1, Ssk1, and Skn7 to accumulate. Dephospho-Skn7 is inactive, whereas dephospho-Ssk1 activates the Hog1 MAP kinase cascade, which stimulates several transcription factors to drive expression of genes to mount the HOG (*H*igh *O*smolarity *G*lycerol) response.

Skn7 associates with GTP-bound Rho1 through an HR1 domain that resides between the N-terminal heat shock factor-like DNA-binding domain and the response regulator domain (4). Although the significance of this interaction has not been tested directly, several lines of genetic evidence from Howard Bussey's laboratory implicate Skn7 in CWI signaling. The *SKN7* gene was isolated initially as a dosage suppressor of the growth defect of a *kre9* mutant (*S*uppressor of *k*re *9*) (7, 34), which is deficient in β1,6-glucan synthesis. Overexpression of *SKN7* suppresses the growth defect of a *pkc1*Δ mutant in the absence of osmotic support (33). However, epistasis experiments suggested that if Skn7 functions within the CWI signaling pathway, its function is likely not under the control of Pkc1. Instead, it was suggested that Skn7 acts in parallel with Pkc1 to regulate cell surface growth. Finally, *MID2*, which encodes a cell surface sensor for CWI signaling (see next section), was isolated as a multicopy activator of an Skn7-LexA-dependent transcriptional reporter (156), suggesting that activation of CWI signaling can stimulate transcriptional activation by Skn7.

Taken together, these data suggest a model in which Skn7 is somehow activated by Rho1 (Fig. 2). However, it is difficult to

hypothesize how Rho1, which is thought to reside only at the plasma membrane, might activate Skn7, which is reported to reside only in the nucleus (33, 129, 192, 266). The Ypd1 phosphorelay protein shuttles between the cytoplasm and the nucleus to phosphorylate Ssk1 and Skn7, respectively, in response to hypo-osmolarity (192). Perhaps a minor pool of Skn7 also undergoes nuclear-cytoplasmic shuttling in response to Rho1 activation.

Skn7 appears to be multifunctional, as reflected by its ability to partner with a variety of other transcriptional regulators at distinct promoter sites under different conditions. For example, its overexpression can bypass the requirement for the cell cycle transcription factors SBF and MBF (223), and it can pair with Mbp1, the DNA-binding component of MBF (31). Skn7 also binds to the Hsf1 heat shock factor and its cognate DNA element (HSE), and possibly the Yap1 oxidative stress factor, in response to oxidative stress (171, 222, 266). However, oxidative stress activation of Skn7 evidently does not involve Sln1 and is independent of the phospho-accepting Asp427 residue (183, 222, 266). Similarly, suppression of *pkc1*Δ is also independent of Asp427 (33). By contrast, Skn7-dependent transcriptional activation of the *OCH1* gene requires phosphorylation of Asp427 through the activity of Sln1 (184). The Skn7 binding site within the *OCH1* promoter is a 13-base-pair repeated sequence, ATTTGGCC/TGGC/GCC, called the Skn7 response element (SSRE), that may reflect a DNA-binding activity intrinsic to Skn7, or may indicate a novel partnering of Skn7 with another transcription factor. The *OCH1* promoter also possesses a functional CDRE that is separate from the SSRE, indicating that Skn7 and calcineurin collaborate in the hypo-osmolarity induction of *OCH1*.

In addition to its function as a transcriptional activator, Skn7 associates with and stabilizes the calcineurin-activated transcription factor Crz1 (355). *SKN7* answered a genetic screen for activators of a CDRE reporter. Intriguingly, its mechanism of action appears to be through interfering with the turnover of Crz1. Phosphorylation of Asp427 is not required for CDRE activation by Skn7, but mutations in either its HR domain or its DNA-binding domain block this function. This raises the interesting possibility that CDRE activation by Skn7 is driven through a Rho1-dependent, Sln1-independent pathway (Fig. 2). The question of the involvement of Rho1 in the regulation of Skn7 awaits further investigation.

Sec3. Cell surface expansion in yeast is driven by polarized exocytosis, a process that involves transport of post-Golgi secretory vesicles along the actin cytoskeleton toward the cell surface. These vesicles dock with components of the exocytic machinery localized to sites of polarized growth and ultimately fuse with the plasma membrane at these sites. A multiprotein complex called the exocyst, which is involved in vesicle targeting and docking at the plasma membrane, assembles at the exocytosis site in response to the arrival of vesicles. Sec3 is a component of the exocyst with the unusual property of localizing to the site of exocytosis independently of active secretion, the actin cytoskeleton, or other components of the exocyst. Therefore, Sec3 is thought to be a spatial landmark for polarized secretion (85).

The two essential G-proteins, Rho1 and Cdc42, have been proposed to be responsible for the spatial regulation of the exocyst complex. Genetic and biochemical evidence connects Sec3 with these GTPases. Sec3 becomes mislocalized in certain *rho1* (*rho1-5* and *rho1-104*) (103) and *cdc42* (*cdc42-13* and *cdc42-201*) (369) mutants. Sec3 associates with Rho1, Rho2, Rho3, and Rho4, as judged by two-hybrid analysis, and with Rho1 and Cdc42, as judged by coprecipitation (103, 369). Moreover, Rho1 and Cdc42 compete in vitro for a direct interaction with the N-terminal domain of Sec3, and an N-terminally truncated form of Sec3 fails to localize in a polarized manner, suggesting that this region of Sec3 may receive targeting information from Rho1 and Cdc42. Therefore, Rho1 and Cdc42 appear to collaborate in the process of vesicle delivery to the plasma membrane through dual control of actin cytoskeleton polarization (for vesicle transport) and vesicle docking through the exocyst. Although a *rho3/4* mutant is not defective in Sec3 localization, Rho3 has been reported to associate with Exo70, another component of the exocyst important for vesicle docking (1, 279). Thus, most members of the Rho family participate at one or more levels in the exocytic pathway.

Cell Surface Sensors: Wsc1-3, Mid2, and Mtl1

A family of five cell surface sensors has been implicated in detecting and transmitting cell wall status to Rho1 (Fig. 1). These include Wsc1 (also called Hcs77 and Slg1) (100, 139, 343), Wsc2 and Wsc3 (343), and Mid2 and Mtl1 (156, 267). A fourth Wsc protein (Wsc4) does not appear to contribute to CWI signaling. These are transmembrane proteins that reside in the plasma membrane (156, 189, 257, 267). Their overall structures are similar in that they possess small C-terminal cytoplasmic domains, a single transmembrane domain, and a periplasmic ectodomain rich in Ser/Thr residues. These Ser/Thr-rich regions are highly O-mannosylated, probably resulting in extension and stiffening of the polypeptide. Therefore, these proteins have been proposed to function as mechanosensors, their ectodomains acting as rigid probes of the extracellular matrix (257, 267). However, there is no direct evidence to support this model.

The Wsc proteins display sequence similarities with one another, (342, 343), and Mid2 shares 50% sequence identity with Mtl1 (156, 267). However, aside from the gross structural similarities between the two subfamilies, their sequences are not conserved. The Wsc proteins possess an N-terminal cysteine-rich domain that is absent from Mid2 and Mtl1. This region, termed the WSC domain, is found in human polycystin 1 (PKD1), a plasma membrane protein that is defective in autosomal dominant polycystic kidney disease (258). A *Trichoderma* β1,3-exoglucanase also possesses a WSC domain (52), suggesting the possibility that this domain binds glucan chains.

Among the constellation of cell wall stress sensors, Wsc1 and Mid2 appear to be the most important. A double *wsc1*Δ *mid2*Δ mutant requires osmotic support to survive (267). The *WSC1* gene was identified through several contemporaneous genetic screens. It was isolated as a dosage suppressor of the temperature-sensitive growth defect of a *swi4*Δ mutant (100). Swi4 is a component of the SBF transcription factor, which is a target of Mpk1 phosphorylation (see below) (196). *WSC1* and *WSC2* were also isolated as dosage suppressors of the heat shock sensitivity of a hyperactive Ras/cyclic AMP pathway mutant (343). The connection to Ras signaling remained obscure until recently, when cell wall defects were discovered to be among

the many phenotypes associated with hyperactive Ras pathway mutants (315).

The isolation of *WSC1* through a screen for mutants that require a constitutive form of *BCK1* (*BCK1-20*) for growth provided a more satisfying link between the cell surface protein and CWI signaling (139). Deletion of *WSC1* results in cell lysis at elevated growth temperatures (e.g., 37 to 39°C), a phenotype that is exacerbated by loss of *WSC2* and/or *WSC3* and suppressed by overexpression of Rho1, Rom2, or Pkc1 (100, 139, 343). Consistent with the importance of Wsc1 for survival of thermal stress, a *wsc1*Δ mutant is deficient in thermal activation of Mpk1 (100, 343). Like most other components of the CWI pathway, Wsc1 localizes to sites of polarized cell growth (63, 129).

The *MID2* gene was isolated initially as a dosage suppressor of the growth defects associated with overexpressed cyclic AMP-dependent protein kinase (Tpk1) (59). This is reminiscent of one of the genetic screens that yielded Wsc1 and supports an antagonistic role for Ras/cyclic AMP pathway signaling in the maintenance of wall integrity. *MID2* also answered a genetic screen for mutants that fail to survive pheromone treatment, a behavior that led to its most widely recognized name (*M*ating *I*nduced *D*eath *2*) (242). *MID2* was additionally isolated as a dosage suppressor of the growth defects associated with loss of the actin-associated protein profilin (203), loss of Wsc1 and Wsc2 (267), and as an activator of the Skn7 transcription factor (156). A *mid2*Δ mutant is not temperature sensitive for growth but is nevertheless somewhat impaired for Mpk1 activation in response to mild heat shock, particularly in combination with *mtl1*Δ (156, 267). Although *MTL1* appears to play a minor role in CWI signaling and has rarely turned up in genetic screens, it was isolated as a dosage suppressor of a temperature-sensitive GS mutant (*fks1-1154 fks2*Δ) (300). Consistent with the importance of Mid2 for the survival of mating, it is required for Mpk1 activation in response to pheromone (156, 267). It should be noted that pheromone-induced activation of CWI signaling is not a direct response to pheromone but rather a secondary response triggered by morphogenesis (35, 81, 276). Further support for the importance of Mid2 in signaling wall stress is its requirement for activation of Mpk1 in response to calcofluor white (156). An interesting distinction between Mid2 and Wsc1 is the former's uniform distribution around the cell periphery (156, 267, 129). The diffuse distribution of Mid2 in the plasma membrane may reflect its role in signaling wall stress resulting from pheromone-induced morphogenesis, which may be initiated at any point on the surface of a G_1-arrested cell.

Despite the differences cited above, it is clear that Wsc1 and Mid2 serve a partially overlapping role in CWI signaling. Overexpression of Wsc1 suppresses the pheromone-induced death associated with *mid2*Δ and, conversely, overexpression of Mid2 suppresses the temperature sensitivity of a *wsc1*Δ mutant (156, 267). Additionally, a double *wsc1*Δ *mid2*Δ mutant displays a severe growth defect, lysing at all temperatures in the absence of osmotic support. The cytoplasmic domains of both proteins display two-hybrid interactions with the N-terminal domain of Rom2 but not with Rho1 (257). This domain is distinct from the Rho1-interacting domain of Rom2, suggesting that the GEF can interact simultaneously with a sensor and with Rho1. Moreover, extracts from *wsc1*Δ and *mid2*Δ cells are deficient in catalyzing GTP loading of Rho1, providing evidence that the function of the sensor-Rom2 interaction is to stimulate nucleotide exchange of Rho1 (257). As would be expected, the short cytoplasmic domains of both Wsc1 and Mid2 are essential to their function (101, 189, 257, 342).

Domain analysis of Wsc1 indicated that the cytoplasmic domain is phosphorylated (189). Further mutational analysis of the cytoplasmic domain revealed that it possesses two short regions important for Rom2 interaction, one at the extreme C terminus and the other near the transmembrane domain (342). The phosphorylation site resides between these interaction regions and serves to inhibit Wsc1 function, probably by interfering with Rom2 interaction. However, phosphorylation is not the only means of Wsc1 regulation. A Wsc1 phosphorylation site mutant (S319/320/322/323A) is not constitutively active but is potentiated for activation by wall stress. It is interesting that a Wsc1 truncation mutant that has only 16 residues of the cytoplasmic domain (the membrane-proximal Rom2 interaction site) retains partial function (342). Such a short region may only be capable of providing a recruitment site for Rom2 rather than imposing a conformational change upon the GEF.

A small plasma membrane-associated protein, called Zeo1, was identified in a two-hybrid screen for interactors with the cytoplasmic domain of Mid2 (101). The Zeo1 protein associates with the region of Mid2 that is closest to the plasma membrane. It evidently interferes with Mid2 signaling because a *zeo1*Δ mutant displays constitutive Mpk1 activity that is dependent on Mid2. Moreover, loss of *ZEO1* suppresses the growth defect of a *rom2*Δ mutant. This suppression likely results from enhanced activity of Mid2 through Rom1. It is not yet clear if Zeo1 interacts with any of the other sensors.

The extensive mannosylation of the periplasmic ectodomains of at least Mid2 and Wsc1 is also important to their function (191, 257). Both proteins are mannosylated by Pmt2 and Pmt4 (191, 257), members of a seven-isoform family of proteins that catalyze the first step in protein O-mannosylation (323). Although there is considerable overlap in substrate specificity among these various mannosyltransferases, only Pmt2 and Pmt4 are capable of modifying Mid2 and Wsc1. Consistently, a double *pmt2*Δ *pmt4*Δ mutant undergoes cell lysis in the absence of osmotic support (97). This defect is suppressed by overexpression of Pkc1, Wsc1, or Mid2 (191), suggesting that O-mannosylation of the sensors, although important, can be bypassed.

It is possible that signal transduction from the cell surface sensors is not unidirectional. Bud tip localization of Wsc1 is disrupted by the actin antagonist latrunculin A, indicating that its positioning is dependent on the actin cytoskeleton. Thus, although Wsc1 controls actin polarization through the action of Rho1, this sensor also responds to changes in the actin cytoskeleton. For this reason, it has been suggested that bidirectional signaling occurs between the actin cytoskeleton and Wsc1 (63) and perhaps the other sensors as well. Such bidirectional signaling is similar to that observed for animal cell integrins, plasma membrane proteins that connect the actin cytoskeleton to the extracellular matrix (298).

Although all five sensors appear to converge on Rho1, there may exist differentially regulated pools of this G-protein at the plasma membrane. The observation that overexpressed Wsc1 but not Mid2 suppresses the growth and GS activity defects of

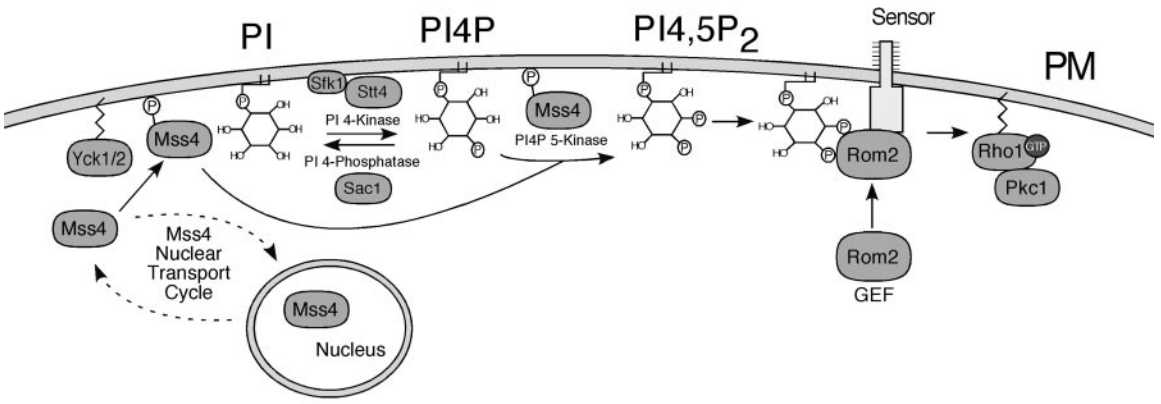

FIG. 4. Phosphoinositide signaling pathway at the plasma membrane (PM). The sequential actions of Stt4 and Mss4 at the cell surface generate $PI4,5P_2$, which recruits Rom2 to the plasma membrane through its PH domain for interaction with the cell surface sensors. The sensors activate Rom2, a GEF for the Rho1 GTPase. Sfk1 appears to be a plasma membrane tethering factor for Stt4. Mss4 cycles between the plasma membrane and the nucleus for reasons that are not yet clear. Yck1 and -2 are plasma membrane-associated casein kinase 1 isoforms that phosphorylate Mss4 and stabilize it at the plasma membrane.

an *fks1*(Ts) *fks2*Δ mutant has prompted the suggestion that Mid2 activates a pool of Rho1 dedicated to the stimulation of Pkc1, whereas Wsc1 can drive activation of multiple Rho1 effectors (300).

Phosphoinositide Metabolism: Stt4-Mss4 Signaling

Phosphoinositide second messengers play an important role in CWI signaling. The first clue to a connection was the isolation of a conditional allele of *STT4*, which encodes an essential phosphatidylinositol 4-kinase (56), through a screen for mutants that displayed both hypersensitivity to the PKC inhibitor staurosporine and temperature sensitivity (*st*aurosporine and *t*emperature sensitive) (363). A *PKC1* mutant presented itself in the same screen (362), and the staurosporine sensitivity of the *stt4*(Ts) mutant was suppressed by overexpression of Pkc1. Additionally, in some genetic backgrounds a null *STT4* mutant could be rescued by high osmolarity (56, 363). *stt4*(Ts) mutants also display defects in actin organization and cell wall integrity (13, 364), providing further support for a connection to CWI signaling.

Stt4 was shown only recently to reside at the plasma membrane, where it catalyzes the synthesis of phosphatidylinositol (PI) 4-phosphate (4P) (11) (Fig. 4). Interestingly, this protein appears to localize to patches at the mother cell cortex. A dosage suppressor of an *stt4*(Ts) mutant, designated *SFK1* (*S*uppressor of *F*our *K*inase) (11), encodes a plasma membrane-localized protein that appears to function as a tethering factor for Stt4. The *SAC1* gene encodes a phosphatidylinositol-4-phosphate, which together with Stt4 regulates the pool of PI4P at the plasma membrane (91). Intriguingly, Sac1 resides predominantly at the endoplasmic reticulum, suggesting the possibility of contact between the peripheral endoplasmic reticulum and the plasma membrane.

Another essential phosphatidylinositol 4-kinase, encoded by *PIK1*, localizes to the nucleus and Golgi (346) and has not been suggested to function in CWI signaling. Indeed, increased expression of either *PIK1* or *STT4* cannot compensate for the loss of the other, suggesting that these phosphatidylinositol 4-kinases both generate an essential pool of PI4P at different membranes (13). Recently, a third phosphatidylinositol 4-kinase, encoded by the nonessential *LSB6* (*L*as *s*eventeen *b*inding) gene, was described as residing at both the plasma membrane and the vacuolar membrane (107). *LSB6* was identified initially as a two-hybrid interactor with Las17, a protein involved in actin patch assembly and actin polymerization (198). Interestingly, overexpression of *LSB6* weakly suppresses the growth defect of an *stt4*Δ mutant (107), suggesting that its function may overlap modestly with that of Stt4.

The *MSS4* gene encodes the only PI4P,5-kinase of yeast, and like *STT4*, it is essential for viability. Mss4 catalyzes the conversion of PI4P at the plasma membrane (the product of Stt4 activity) to $PI4,5P_2$ (70, 122). This gene was isolated initially in a screen for dosage suppressors of the temperature sensitivity of an *stt4*(Ts) mutant (364). Similarly to *stt4*(Ts) mutants, *mss4*(Ts) mutants display actin organization defects and cell lysis at the restrictive temperature (12, 70). Audhya and Emr (11) identified Rom2 as the first effector of the Stt4-Mss4 pathway by demonstrating that a critical role of $PI4,5P_2$ production is to recruit this Rho-GEF (and presumably Rom1) to the plasma membrane through its plekstrin homology (PH) domain. This recruitment is evidently integral to the activation of Rom2 GEF activity for Rho1 (Fig. 4). Interestingly, levels of $PI4,5P_2$ increase transiently in response to mild heat shock (11, 70), a stress that activates Mpk1 (148), suggesting that the concentration of this phosphoinositide in the plasma membrane contributes to the stress-induced activation of CWI signaling.

Although initial immunofluorescence studies localized Mss4 to the plasma membrane (122), a more recent study demonstrated that this protein shuttles between the nucleus and the plasma membrane (12). A mutant form of Mss4 that does not exit the nucleus was nonfunctional, but its function was restored by artificially tethering it to the plasma membrane, confirming that the essential function of this lipid kinase is at the cell surface. The purpose of Mss4 nuclear-cytoplasmic shuttling is not clear, but it may have a nonessential nuclear role. Mss4 is also phosphorylated at the plasma membrane by the casein kinase I isoforms encoded by *YCK1/2* (12). This

phosphorylation is required for stable membrane association of Mss4. It is possible that Yck1 and -2 are regulated by inputs that activate phosphatidylinositol signaling at the plasma membrane. However, these protein kinases are involved in diverse processes, including morphogenesis, cell wall biogenesis, nutritional signaling, cytokinesis, phosphoinositide metabolism, pheromone response, endocytosis, and turnover of membrane proteins (12, 120, 202, 224, 277, 278), suggesting that they may act simply by virtue of proximity to targets that present themselves at the plasma membrane.

Several additional targets of Stt4-Mss4 signaling have been suggested recently. The Cla4 protein kinase, which is a direct effector of the Cdc42 GTPase and is important for polarized cell growth, possesses a PH domain that binds in vitro to several phosphoinositide species (354). A mutant in *STT4* but not *MSS4* was defective in localization of Cla4 to the bud tip, suggesting that plasma membrane PI4P is responsible for recruitment of Cla4 to the cell surface for activation by Cdc42. Boi1 and Boi2, two related proteins of undetermined function, have also been suggested as targets of Cdc42 action that are important for polarized cell growth (25). Significantly, Boi1 and -2 possess $PI4,5P_2$ -binding PH domains (106), suggesting that, like Cla4, these proteins may be recruited to the plasma membrane for interaction with Cdc42. Finally, a recent genomewide synthetic lethality screen with an *mss4*(Ts) mutant yielded a pair of essential PH-domain proteins, designated Slm1/2 (*S*ynthetic *l*ethal with *m*ss4) (14). Slm1 and -2 are required for polarization of the actin cytoskeleton, and their localization to the plasma membrane requires $PI4,5P_2$. The lethality of an *slm1*Δ *slm2*Δ mutant was suppressed by constitutive activation of Rho1 or Pkc1. Interestingly, Slm1 and -2 are also targets for Tor2 protein kinase activity and may be at least partially responsible for regulation of the actin cytoskeleton by Tor2 (see Tor signaling below).

Nuclear Targets of Mpk1

At present, only a few targets have been established for the Mpk1 MAP kinase, including two transcription factors, a protein phosphatase that reciprocally regulates Mpk1 and a cell surface Ca^{2+} channel. Genetic evidence has implicated Mpk1 in the control of several additional substrates, including the actin cytoskeleton. However, which specific cytoskeletal protein or proteins are substrates for Mpk1 is not clear. For that reason, only processes for which a specific protein is known or suspected to be an Mpk1 substrate are discussed below.

Rlm1. The Rlm1 transcription factor (for *r*esistant to the *l*ethality of *M*kk1^{S386P}) is responsible for the majority of the transcriptional output of CWI signaling. The *RLM1* gene was identified in a genetic screen for mutants that could survive the growth inhibition caused by overexpression of a constitutive form of Mkk1 (350, 361). The encoded factor possesses an N-terminal DNA-binding domain related to the MADS (*M*CM1, *A*gamous *D*eficiens, *S*erum response factor)-box family of transcriptional regulators. Rlm1 is most closely related to mammalian MEF2, sharing the same in vitro binding specificity ($CTA[T/A]_4TAG$) (72). However, in vivo studies revealed that the binding specificity is relaxed at the terminal G/C base pairs (142, 143). Rlm1 displays two-hybrid interaction with Mpk1 (349) and is phosphorylated in vivo and in vitro by Mpk1 (359). Rlm1 evidently always resides in the nucleus, but its phosphorylation by Mpk1 at two residues within its transcriptional activation domain (Ser427 and Thr439) stimulates its activity (143). A MAP kinase docking site in the activation domain (delimited by residues 324 to 329) is shared with MEF2 and is also essential for activation by Mpk1 (143).

A genomewide survey of gene expression through CWI signaling revealed that Rlm1 regulates the expression of at least 25 genes, most of which encode cell wall proteins or have been otherwise implicated in cell wall biogenesis (144). This analysis was conducted using a constitutively active form of Mkk1 (Mkk1^{S386P}) expressed under the inducible control of the *GAL1* promoter so as to restrict the output to genes that are regulated specifically by this pathway. Remarkably, regulation of the expression of all these genes (both positive and negative) in response to cell wall stress required Rlm1, indicating that this factor can act as either a transcriptional activator or a repressor depending on the context. The 20 genes identified as being activated in response to Mkk1^{S386P} expression were also activated by mild heat shock in an Rlm1-dependent manner. A similar global gene expression study reported the use of constitutive forms of Pkc1 and Rho1 to identify an overlapping set of CWI signaling-regulated genes (276). In this study, *RLM1* was identified among the induced genes, suggesting the existence of an autoregulatory circuit for amplification of the stress response. Consistent with this, the *RLM1* gene was also induced in response to cell wall stress associated with an *fks1*Δ mutation (36).

One intriguing transcriptional target of Rlm1 is *MLP1* (Mpk1-like protein kinase), which encodes a homolog of Mpk1 (350). Its function has remained shrouded, due largely to an absence of phenotypic defects associated with its loss. Mlp1 shares 53% sequence identity with Mpk1, interacts with Rlm1 by two-hybrid analysis, but is lacking several catalytic domain residues recognized to be critical for protein kinase activity. First, the threonyl residue within the dual phosphorylation site of the activation loops of MAP kinases (T-X-Y) is mutated to Lys in Mlp1, indicating that it is not a true MAP kinase. Second, a universally conserved Lys residue within the ATP-binding site of all protein kinases is mutated to Arg. This is a mutation often generated to create a catalytically inactive form of protein kinases. Third, a universally conserved Asp residue within the triplet DFG in subdomain VII is mutated to Asn. Although Mlp1 protein levels increase by approximately 100-fold in response to cell wall stress, efforts to detect protein kinase activity have not been successful (A. Sobering and D. E. Levin, unpublished). It seems likely that Mlp1 is a pseudokinase that competes with Mpk1 for access to either Rlm1 or Mkk1/2.

Unlike loss of components of the MAP kinase cascade, deletion of *RLM1* does not result in temperature-dependent cell lysis (349). Instead, an *rlm1*Δ mutant displays caffeine sensitivity, a phenotype that is shared with mutants defective in the MAP kinase cascade. The modest phenotypic defect of an *rlm1*Δ mutant compared with an *mpk1*Δ mutant suggests that Mpk1 phosphorylates additional targets. A second MEF2-like transcription factor, Smp1, displays in vitro binding specificity similar to that of Rlm1 and can form heterodimers with Rlm1 in vitro but is evidently not involved in CWI signaling (72). In fact,

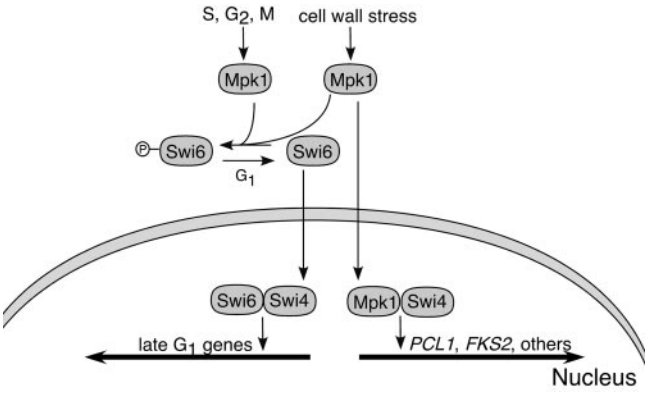

FIG. 5. Model for the regulation of Swi4 by Mpk1. Mpk1 is activated both by cell wall stress and periodically through the cell cycle. Swi6 is the regulatory subunit that complexes with Swi4 to form SBF, a cell cycle-specific transcription factor. During periods other than G_1, Swi6 is phosphorylated on Ser160, which causes its exclusion from the nucleus. It is likely that this phosphorylation is catalyzed by Mpk1. Mpk1 also phosphorylates Swi6 in response to cell wall stress. Swi4, the DNA-binding component of SBF, associates with Mpk1 in vitro and may form an alternative transcriptional complex for the regulation of some cell wall- and morphogenesis-related genes, notably *FKS2* and *PCL1*.

recent evidence indicates that Smp1 is a direct target of the Hog1 MAP kinase in response to high external osmolarity (65).

SBF (Swi4/Swi6). The Cln3-Cdc28 cyclin/cyclin-dependent kinase (CDK) complex drives gene expression in late G_1 phase through the related transcription factors SBF and MBF (reviewed in reference 32). These factors share a common regulatory subunit, Swi6, and one of two related DNA-binding proteins—Swi4 in SBF and Mbp1 in MBF. Additionally, recent reports have identified Whi5 as an inhibitory subunit of SBF and possibly MBF (54, 61). SBF binds the upstream regulatory sequence CACGAAA (SCB; for *S*wi4/6-dependent cell *c*ycle *b*ox) during G_1 phase and regulates both the transition from G_1 to S (referred to as Start) and cell morphogenesis. SBF is required for the maximal G_1-specific expression of *CLN1*, *CLN2*, *PCL1*, and *PCL2*, which encode G_1 cyclins (32, 220, 241), and several genes involved in cell wall biosynthesis (130, 318). Cln1 and Cln2 associate with Cdc28, whereas Pcl1 and Pcl2 associate with the Pho85 CDK (214). Binding of SBF to SCBs during early G_1 is not sufficient for transcriptional activation at Start (110, 161). SBF-mediated transcription is activated by phosphorylation of Whi5 by Cln3-Cdc28, which triggers its release from Swi4/Swi6 (54, 61). Swi6 is also phosphorylated (on Ser160) in a cell cycle-dependent manner but not by Cdc28 (309). Phosphorylation of Swi6 on Ser160 interferes with an adjacent nuclear import signal and results in cytoplasmic localization of this factor at times other than G_1 (Fig. 5).

Although a critical function of SBF is to drive G_1-specific transcription, it has been suggested that this factor has a role outside of G_1 (18, 196). Based on genetic and biochemical evidence, Mpk1 has been proposed to regulate SBF in response to cell wall stress. The temperature-dependent cell lysis defect of an *mpk1*Δ mutant is suppressed by overexpression of Swi4 (but not Mbp1 or Swi6) or by overexpression of the Pcl1 or Pcl2 (but not Cln1 or Cln2) cyclin (130, 196). Similar to *mpk1*Δ mutants, *swi4*Δ (but not *swi6*Δ) cells display osmotic temperature-sensitive growth in some strain backgrounds, although *swi4*Δ cells arrest growth in G_1 at the restrictive temperature without undergoing cell lysis (196, 241). Both *swi4*Δ and *swi6*Δ mutants are hypersensitive to calcofluor white, supporting a role for SBF in cell wall biogenesis (130).

Mpk1 associates with SBF in vivo, as judged by coprecipitation experiments (18, 196). Moreover, Swi6 is phosphorylated in vitro by Mpk1 and in vivo in an Mpk1-dependent manner in response to cell wall stress (i.e., shift to 39°C or treatment with mating pheromone) (196). It is interesting that the cell cycle-regulated phosphorylation site of Swi6 (Ser160) resides within a consensus MAP kinase phosphorylation site (i.e., S/T-P) and a mutant form of Swi6 that lacks this site is not phosphorylated by Mpk1 (18). This suggests the possibility that Mpk1 may also regulate SBF through the cell cycle. Consistent with this notion is the fact that Mpk1 activity fluctuates through the cell cycle, peaking in G_1 (368). Because Swi6 is required for the cell cycle-dependent binding of Swi4 to DNA (17, 110, 161), Mpk1 may regulate SBF activity in part by driving Swi6 out of the nucleus both during periods of the cell cycle in which SBF is inactive and under conditions of wall stress.

Swi4 may also be a phosphorylation target for Mpk1 in vitro, but the phosphoprotein detected in the only such report did not migrate as would be expected for Swi4 (196). Perhaps more interesting is the finding that Mpk1 can associate specifically with Swi4 in vitro (18), suggesting a model in which an Mpk1/Swi4 dimer is an active transcription factor in the absence of Swi6. Indeed, transcriptional induction of at least two genes, *PCL1* and *FKS2*, in response to mild heat shock depends on both Swi4 and Mpk1 but not Swi6 (18; D. E. Levin, K. Y. Kim, and U. S. Jung, unpublished) (Fig. 5). Moreover, chromatin immunoprecipitation experiments revealed that Swi4 is able to bind to the promoters of a subset of Swi4-dependent genes, including *PCL1*, in the absence of Swi6 (18). In further support of this model is the observation that Mpk1 possesses some transactivation activity (317).

As noted above, *FKS2* expression is regulated not through the cell cycle but in response to cell wall stress, supporting a role for Swi4 outside of its recognized function in G_1. Equally intriguing is the recent demonstration that Pcl1 and Pcl2 localize to sites of polarized cell growth (220), an unprecedented behavior for cyclins. A role for G_1 cyclins in establishing or maintaining cell polarity may explain both the function of Swi4 in morphogenesis and the suppression of *mpk1*Δ mutants by overexpressed Pcl1 or Pcl2.

The temperature-sensitive growth defect of an *mpk1*Δ mutant is also suppressed by overexpression of a poorly characterized protein called Bck2 (for *B*ypass of *C*-*k*inase) (172). As suggested by its name, Bck2 overexpression also suppresses the growth defect of a *pkc1*Δ mutant. Although unrelated by sequence, Bck2 serves a shared function with Cln3 in the late G_1 induction of SBF target gene expression (71, 80). In *cln3*Δ and *bck2*Δ mutants, expression of SBF-regulated genes is delayed, resulting in a delay at Start and an attendant large-cell phenotype. A *cln3*Δ *bck2*Δ mutant is inviable but can be suppressed by overexpression of Cln2, either directly or through excess expression of Swi4 (80, 353). Additionally, overexpression of Bck2 drives SBF target gene expression in a manner that is partially dependent on Swi4 and Swi6 but independent of Cdc28 (71, 353). Therefore, it seems likely that the mechanism

by which Bck2 overexpression suppresses loss of Mpk1 function is shared with that of Swi4—enhanced levels of Pcl1 and Pcl2. However, a deeper understanding of Bck2 function and regulation awaits its molecular analysis.

Cytoplasmic Targets of Mpk1

The growth defect of a double *rlm1*Δ *swi4*Δ mutant is less severe than that of an *mpk1*Δ mutant, and the former does not appear to have difficulties with actin polarization (D. E. Levin, unpublished), suggesting that additional Mpk1 targets exist. Consistent with this interpretation, a small pool of Mpk1 localizes to sites of polarized growth (341), implying that it has targets at the cell surface. Moreover, nuclear Mpk1 relocalizes to the cytoplasm in response to cell wall stress (148), presumably to activate extranuclear targets. Some of these have been described recently and are summarized below. In addition to these potential targets, Mpk1 has been proposed to drive polarization of the actin cytoskeleton (212).

Cch1/Mid1 Ca^{2+} channel. *Saccharomyces cerevisiae* possesses a high-affinity Ca^{2+} influx system in its plasma membrane comprised of at least two subunits, Cch1 and Mid1. Cch1 is related to the α1 pore-forming subunit of L-type mammalian voltage-gated Ca^{2+} channels (86, 251). Mid1 does not appear to have a mammalian homolog but displays stretch-activated Ca^{2+}-permeable channel activity when expressed in animal cells (150, 151). The two proteins have been proposed to form a common Ca^{2+} influx system based on nearly identical phenotypic defects associated with loss of their respective functions and the observation that Mid1 coimmuneprecipitates with Cch1 (29, 188, 227). Activation of the Cch1-Mid1 channel results in accumulation of intracellular Ca^{2+} and activation of calcineurin, the Ca^{2+}- and calmodulin-dependent serine/threonine-specific protein phosphatase (Fig. 2). Stimuli that cause activation of Cch1-Mid1 and calcineurin include pheromone treatment (57, 89, 131, 226), mild heat shock (371), hypo-osmotic shock (23), endoplasmic reticulum stress (29), and an increase in extracellular cations (i.e., Li^+ and Na^+) (10, 216, 230, 284). Thus, at least three types of cell surface proteins are activated in response to a hypo-osmotic shift—the Cch1/Mid1 channel, the Sln1 osmosensor, and the CWI sensors.

Calcineurin dephosphorylates several targets, including the Crz1/Tcn1 transcription factor (207, 320), which allows entry of this factor into the nucleus (321). Calcineurin also inhibits the Cch1/Mid1 channel in what appears to be a negative feedback loop (29, 188, 227). Recent evidence indicates that a target of calcineurin action is the Cch1 subunit (28). Moreover, a screen of protein kinase knockout mutants revealed that Mpk1 is required for activation of the Cch1-Mid1 channel in response to endoplasmic reticulum stress (which may cause cell wall stress indirectly), suggesting that calcineurin antagonizes CWI signaling in this instance (28). It will be interesting to determine if agents that specifically induce cell wall stress result in phosphorylation and activation of the Cch1/Mid1 channel. If so, there exist at least three points of interaction between the CWI signaling pathway and Ca^{2+} signaling: activation of the Cch1/Mid1 channel by Mpk1, activation of Crz1 by Rho1-Skn7 (4, 355), and collaboration between Mpk1 and Crz1 to activate *FKS2* expression in response to cell wall stress (371). These interactions are summarized in Fig. 2.

MAP kinase phosphatases. Mpk1 is downregulated by four distinct protein phosphatases. Interestingly, two of these, Msg5 and Ptp2, appear to undergo reciprocal regulation by Mpk1. The *MSG5* gene, which encodes a dual-specificity (Tyr and Ser/Thr) protein phosphatase, was first identified through its ability to downregulate Fus3 (73). However, it was also isolated as a dosage suppressor of the growth defect associated with overexpression of a constitutive allele of *MKK1* ($Mkk1^{P386}$) (349). Deletion of *MSG5* also results in increased basal activity of Mpk1 (205). A recent report revealed that Msg5 and Mpk1 act on each other in vivo and in vitro (87). Mpk1 phosphorylates Msg5 in response to CWI pathway activation. A decrease in affinity between Msg5 and Mpk1, as judged by coprecipitation, was observed after activation of Mpk1 by mild heat shock, suggesting that Mpk1 phosphorylation of Msg5 interferes with association between the proteins. This would constitute a positive feedback loop for prolonged activation of Mpk1, which has been observed in response to thermal upshift (148). The function of Msg5 with respect to CWI signaling may therefore be to maintain a low basal activity of Mpk1 in the absence of stress.

The Ptp2 and Ptp3 tyrosine phosphatases, which have been shown to dephosphorylate the Fus3 and Hog1 MAP kinases, also act on Mpk1 in vivo and in vitro (209). Both genetic and biochemical evidence suggests that Ptp2 is more effective than Ptp3 against activated Mpk1. Additionally, expression of *PTP2* but not *PTP3* is induced in response to mild heat shock in an Mpk1-dependent manner, suggesting that activation of Mpk1 establishes a negative feedback loop for its inactivation by Ptp2. Induction of *PTP2* expression is partially under the control of Rlm1 (105). The positive regulation of *PTP2* expression by Mpk1 is in contrast to its negative regulation of Msg5 activity. Perhaps Ptp2 and Ptp3 function to reestablish the resting state of Mpk1 after stress-induced activation.

Finally, the dual-specificity protein phosphatase Sdp1 appears to target Mpk1 specifically. Two-hybrid and coimmunoprecipitation analyses suggest that among the five MAP kinases in yeast, Mpk1 is the only one with which Sdp1 interacts (53). Additionally, overexpression of either wild-type *SDP1* or a dominant negative allele has opposite effects on Mpk1 activity. Like multicopy *MSG5*, overexpression of *SDP1* suppresses the growth defect of cells expressing constitutive *MKK1* (105). Expression of *SDP1* is under the control of the Msn2/4 stress-activated transcription factors. Thus, although Sdp1 may be the only protein phosphatase dedicated solely to the regulation of Mpk1, its regulation appears to be independent of Mpk1.

Mih1 tyrosine phosphatase: morphogenesis checkpoint. *Saccharomyces cerevisiae* undergoes a mitotic delay in response to perturbation of the actin cytoskeleton (213, 307). This delay, known as the morphogenesis checkpoint, is mediated by the inhibitory action of the Swe1 tyrosine kinase on the Cdc28 CDK (182). Phosphorylation of Cdc28 is reversed by the action of the Mih1 tyrosine phosphatase (the budding yeast homolog of mammalian Cdc25). Activation of the morphogenesis checkpoint results in cell cycle arrest in G_2 phase until the actin cytoskeleton can be repolarized to complete bud construction (181). An *swe1*Δ mutant fails to arrest in response to disruption of the actin cytoskeleton, proceeding through mitosis in the absence of bud formation to yield binucleate cells. CWI signaling was implicated in the morphogenesis checkpoint by the

finding that an *mpk1*Δ mutant is deficient (though not completely defective) in G_2 arrest induced by the actin antagonist latrunculin B (111). Genetic evidence suggests that Mpk1 promotes checkpoint activation not by enhancing Swe1 activity but by downregulating the Mih1 phosphatase. Specifically, an *mih1*Δ mutation restores checkpoint function to an *mpk1*Δ mutant. However, it is not yet clear if Mih1 is a direct target of Mpk1.

ACTIVATION OF CWI SIGNALING

The CWI pathway is regulated through the cell cycle but is also activated in response to a variety of external stimuli that cause cell wall stress. Signaling through the CWI pathway is typically monitored by any of three approaches. The most direct method is to follow the activation state of Mpk1. The protein kinase activity of epitope-tagged Mpk1 can be measured in immune complexes using bovine myelin basic protein as a substrate (148, 368). Alternatively, because Mpk1 is activated by phosphorylation of neighboring threonyl and tyrosyl residues within its activation loop, analogous to Thr^{202}/Tyr^{204} of mammalian p44/p42 MAP kinase (Erk), commercially available antibodies against phospho-p42/p44 are quite effective at detecting activated Mpk1 (66, 205). A less direct but simpler method of measuring sustained signaling through the CWI pathway employs *lacZ* reporters driven by Rlm1-responsive promoters. A set of highly sensitive reporter plasmids have been described, some of which are induced 100-fold over several hours in response to cell wall stress (143).

Cell Cycle Regulation

The earliest suggestion that Mpk1 was involved in cell cycle regulation was a report that mutations in *MPK1* displayed additive growth defects with a mutant allele in *CDC28*, which encodes the CDK of cell cycle engine (212). These authors also isolated *MPK1* as a dosage suppressor of another *cdc28* allele. Characterization of the *mpk1* mutants revealed that they display defects in polarized growth in response to mild heat shock, including delocalization of cortical actin patches and accumulation of secretory vesicles (212). From these results, the authors proposed that Mpk1 functions either downstream of or in parallel to Cdc28 in promoting bud emergence.

Later work revealed that CWI signaling is regulated periodically through the cell cycle, peaking at the time of bud emergence, the time at which growth is most highly polarized (368). Mpk1 activation occurs at a time coincident with bud emergence in cells synchronized by treatment with α-factor. By contrast, Mpk1 is inactive in cells blocked in mitosis by nocodazole. This makes sense from the perspective of cell wall remodeling through the cell cycle. At times when growth is polarized to a single site on the cell surface, the cell experiences the greatest wall stress and is most vulnerable to lysis. By contrast, during G_2 and mitosis, cell surface growth becomes isotropic. Indeed, conditional mutants in *PKC1* arrest with small buds (177, 180, 253). Electron micrographic studies revealed that *pkc1*-arrested cells had lysed at their bud tips, the site of wall remodeling (178). Although Mpk1 activity is regulated through the cell cycle, its activation is not strictly dependent on Cdc28 (368). Cyclic Mpk1 activity probably reflects the level of cell wall stress signaled during different parts of the cell cycle. Consistent with this interpretation, the localization of CWI pathway components reveals that most follow a cell cycle-dependent pattern of localization to sites of polarized cell growth.

Heat Stress

CWI signaling is induced in response to several environmental stimuli. First, signaling is activated persistently in response to growth at elevated temperatures (e.g., 37 to 39°C) (148, 368), consistent with the finding that null mutants in many of the pathway components display cell lysis defects only when cultivated at high temperature. Interestingly, Mpk1 is not activated immediately upon heat shock. Activation is detectable after approximately 20 min and peaks after 30 min (148). This suggests that the signaling pathway is not sensing the temperature change directly but is detecting some secondary effect of exposure to high temperature. One response to thermal stress is the accumulation of cytoplasmic trehalose (235), which can reach levels of greater than 0.5 M for the purpose of protecting proteins from thermal denaturation (125, 310, 311). As will be discussed below, such a drastic increase in intracellular osmolarity would increase the turgor pressure, which may be the stress that the CWI pathway detects at high temperature. In any case, the cell surface sensors are important for thermal activation of Mpk1 (100, 156, 205, 267).

Heat shock may contribute to Mpk1 activation through an additional input at the level of the MAP kinase. Although null mutants in *PKC1*, *MKK1/2*, and *BCK1* are blocked for activation of Mpk1 by all tested forms of wall stress, including heat shock (35, 60, 112, 148), a *pkc1*Δ mutant that is kept alive by a constitutive allele of *BCK1* is capable of activating Mpk1 in response to mild heat shock (112). Likewise, heat shock activates Mpk1 in a *bck1*Δ mutant expressing a constitutive form of Mkk1. These findings led to the proposal that the protein kinase cascade is required in a passive way to provide basal signal to the MAP kinase and that heat shock activation is achieved by inhibition of the protein phosphatases that act on Mpk1 (i.e., Sdp1, Msg5, Ptp2, and Ptp3). However, this model ignores the observation that Pkc1 is activated in vivo by heat shock (134). Moreover, elimination of the MAP kinase phosphatases does not abrogate heat shock activation of Mpk1 (112). Therefore, it seems likely that there are two inputs driving the response to this particular form of wall stress—one through the sensors at the top of the pathway to send an activating signal to Mpk1, and a second that inhibits the MAP kinase phosphatases. This second pathway appears to be specific to heat shock activation of Mpk1 (112).

Hypo-osmotic Shock

Hypo-osmotic shock induces a rapid but transient activation of CWI signaling (60, 148). Mpk1 is activated within 15 seconds of an osmotic downshift, but basal activity is restored after approximately 30 minutes. A further osmotic downshift at this point reactivates Mpk1, indicating that the cell senses a change in osmotic potential rather than sensing solute concentration. As discussed above, the Sln1 cell surface osmosensor is also activated by hypo-osmotic shock, which results in activation of the Skn7 transcription factor in support of cell wall biogenesis.

By contrast, the Hog1 MAP kinase is activated in response to hyperosmotic shift (a result of Sln1 inactivation). In addition to these pathways, the Cch1/Mid1 Ca^{2+} channel is activated by hypo-osmotic shock, which results in activation of the Crz1 transcription factor. That all three of these signaling pathways are involved in the cell wall response to hypo-osmotic shock is a testament to the challenge posed by this particular stress to the survival of the yeast cell.

Pheromone-Induced Morphogenesis

Haploid yeast cells constitutively secrete mating pheromones. Detection of the presence of pheromone from cells of the opposite mating type triggers a developmental program through the mating pheromone response pathway (79). This pathway induces cell cycle arrest in G_1 phase followed by the formation of a mating projection toward the source of pheromone. Projection formation requires polarization of the actin cytoskeleton and the secretory pathway. Treatment with mating pheromone stimulates CWI signaling at a time that is coincident with the onset of projection formation (35, 81, 368). Indeed, mutants defective in CWI signaling undergo cell lysis during pheromone-induced morphogenesis (81), reflecting the major reorganization of the cell wall associated with projection formation.

It is important to note that pheromone-induced CWI signaling is an indirect event linked to morphogenesis. Mutants blocked in pheromone response also fail to activate Mpk1 after treatment with mating pheromone (35). Moreover, mutants in *SPA2* or *BNI1*, which exhibit a delay in pheromone-induced morphogenesis, also delay activation of Mpk1. Similar to CWI signaling, calcineurin is activated as a late response to pheromone treatment (356), probably as a consequence of morphogenesis-induced cell wall stress. Another similarity is the requirement of calcineurin for survival of pheromone treatment (356).

Rho1 localizes to projection tips of cells treated with pheromone (15, 21). The Gβγ complex of the pheromone response pathway, which provides the positional clues for polarity establishment, recruits Rho1 to the site of polarized growth (21). Rho1 associates in vivo with the Gβ subunit (Ste4) as judged by affinity precipitation experiments. Precedents for the association of mammalian RhoA with Gβ subunits reveal that this interaction is highly conserved (4, 108). Pkc1 is also recruited to the tips of mating projections in a manner dependent on Rho1 association with Gβ, presumably reflecting the recruitment of Pkc1 to GTP-bound Rho1 (21).

Cell Wall-Stressing Agents

Agents that cause cell wall stress, such as the chitin antagonist calcofluor white, the β1,3-glucan-binding dye Congo red, the cell wall lytic enzyme zymolyase, and caffeine, activate CWI signaling (66, 156, 143, 156, 165, 205). The mechanism by which caffeine induces wall stress is not understood, but a recent genome profiling study suggests that this drug may target the Tor protein kinase complexes (193). Mutations that impair cell wall biosynthesis (e.g., *fks1*Δ) also activate signaling (36, 327). The nature of the wall stress seems to be unimportant, suggesting that any treatment that interferes with maintenance of cell wall integrity is sufficient to trigger signaling. A genomewide analysis of genes upregulated by mutations that affect the cell wall (i.e., *fks1*, *gas1*, *kre6*, *mnn9*, and *knr4*) identified a group of approximately 80 upregulated genes common to these wall-stressing mutations. In silico analysis of the regulatory regions of these genes revealed that many possess sites for Rlm1, Swi4, and Crz1 as well as Msn2/4 and Hsf. Similar analyses using Congo red, zymolyase, or calcofluor white to induce wall damage have identified an overlapping set of genes that implicated the same group of transcription factors (30, 95). These results are consistent with the coactivation of CWI signaling and Ca^{2+} signaling as well as general stress signaling under these conditions.

Actin Cytoskeleton Depolarization

Rapamycin treatment, which depolarizes the actin cytoskeleton by specifically inhibiting the shared function of the Tor1/2 protein kinases, induces Mpk1 activation (166, 333). Direct depolarization of the actin cytoskeleton by treatment with the actin antagonist latrunculin B also activates Mpk1 (111). Although Rho1 and Pkc1 are required for Mpk1 activation in response to actin destabilization, there is disagreement as to the requirement for the cell surface sensors. Torres et al. (333) found that mutants in *WSC1* and especially *MID2* were deficient in Mpk1 activation in response to rapamycin treatment (1 h). By contrast, Krause and Gray (166) found little effect of these mutations on Mpk1 activation after a longer (2 h) rapamycin treatment. Consistent with the conclusion of the latter study, Harrison et al. (111) reported that treatment with the actin antagonist latrunculin B activated Mpk1 independently of the sensors, suggesting a novel activation mechanism that nevertheless requires the protein kinase cascade. However, Mpk1 activation by either treatment was blocked (333) or appreciably diminished (111) by osmotic support, suggesting that the CWI pathway senses cell wall stress induced by these actin antagonists. Clearly, more work is required to establish the route by which Mpk1 is stimulated in response to disruption of the actin cytoskeleton.

Oxidative Stress

Lipid peroxidation induced by linoleic acid hydroperoxide activates Mpk1 (6), although the mechanism has not been explored. Perhaps oxidation of the plasma membrane generates a cell wall stress signal. An intriguing recent report suggests that oxidative stress induced by hydrogen peroxide results in phosphorylation of Ask10 in a manner dependent upon Mkk1/2 but not Mpk1 (51). Ask10 mediates the oxidative stress-induced destruction of the C-type cyclin Srb10, which is important for repression of several stress response genes. This study suggested that Mkk1/2 might have an alternative target to Mpk1 in response to oxidative stress. If true, this would be unprecedented behavior among MEKs, which are believed to specifically phosphorylate their cognate MAP kinases. Interestingly, Ask10 was identified initially in a genetic screen for dosage activators of Skn7 (250). Because one function of Skn7 is to mediate some aspect of the oxidative stress response (167, 183, 222, 312), Ask10 may be a clue to the largely unexplored link between CWI signaling, Skn7, and oxidative stress.

Plasma Membrane Stretch

A simplifying concept to explain the multitude of seemingly different stimuli that activate CWI signaling is that there is a common stress generated by all activating conditions. Evidence is accumulating that plasma membrane stretch is the underlying physical stress that leads to activation of CWI signaling. Chlorpromazine, an amphipathic molecule that causes membrane stretch by asymmetric insertion into the plasma membrane, is a potent activator of Mpk1 (148). In further support of this model is the observation that increased extracellular osmolarity, which blocks outward plasma membrane stretch by neutralizing turgor pressure, prevents activation of CWI signaling by various wall stressors (66, 111, 148, 333).

Another line of supportive evidence concerns the genetic link between the Ppz1 and -2 protein phosphatases and the CWI pathway (172, 260). Overexpression of either *PPZ1* or *PPZ2* suppresses the cell lysis defect of an *mpk1*Δ mutant. Deletion of both *PPZ1* and *PPZ2* results in an osmoremedial cell lysis defect, and *ppz*Δ mutants display additive defects with *mpk1*Δ.

This genetic interaction was traced recently to the role of the Ppz phosphatases in regulating K^+ homeostasis (217). The intracellular concentration of K^+ is a major determinant of turgor pressure in living cells. Mutants defective in *PPZ1/2* accumulate increased intracellular K^+ and display constitutive CWI signaling, supporting the notion that this signaling pathway senses turgor pressure, perhaps by monitoring plasma membrane stretch (217). Finally, mild heat shock results in the accumulation of trehalose, which, in addition to protecting proteins from thermal denaturation, also increases the intracellular osmolarity (310, 311). The associated increase in turgor pressure is likely to be responsible for activation of CWI signaling. During periods of polarized cell growth, cell wall expansion at bud tips and mating projections may be a natural source of plasma membrane stretch. However, it is not yet clear by what mechanism the cell surface sensors would detect membrane stretch.

Delocalization of Signaling Components

When cells are subjected to cell wall stress, the actin cytoskeleton and many of the proteins of the CWI signaling pathway that are normally localized to sites of polarized growth become redistributed around the cell periphery or to specific regions underlying the cell surface (8, 63). Although only Rho1, Fks1, Wsc1, and Pkc1 have been examined, it is likely that other components are similarly redistributed. This stress-induced relocalization of the upper components of the CWI signaling pathway has been proposed to be a homeostasis mechanism to repair cell wall damage (63). By this model, generalized cell wall stress, caused by such agents as heat shock or zymolyase treatment, causes transient delocalization of the actin cytoskeleton. As a consequence, the GS and other Rho1 effectors are also redistributed to deal with the emergent damage.

ALTERNATIVE Pkc1 PATHWAY BRANCHES

Loss of *PKC1* function results in cell lysis at all growth temperatures in the absence of osmotic support (177, 178, 253). However, loss of any of the components of the MAP kinase cascade under the control of Pkc1 results in cell lysis only under conditions of cell wall stress, such as at elevated growth temperatures (137, 173, 174, 204). The increased severity of the *pkc1*Δ defect led to the proposal that Pkc1 regulates other targets in addition to Bck1 (174, 178). Some additional substrates of Pkc1 have been identified biochemically, and some target pathways have been suggested by genetic interactions with Pkc1. These are summarized below.

Cell Wall Targets of Pkc1

Oligosaccharyl transferase. The *STT3* gene was one of several identified in a genetic screen for mutants that were sensitive to the PKC inhibitor staurosporine and to high temperature (362). Pkc1 is the target of staurosporine action in yeast (362, 348). Stt3 is an essential subunit of the oligosaccharyl transferase (OT) complex, which catalyzes protein *N*-glycosylation in the lumen of the endoplasmic reticulum (365, 373). Because protein *N*-glycosylation is important for cell wall assembly, it was reasonable to posit that mutants deficient in this process might not be able to tolerate a deficiency in CWI signaling caused by staurosporine (373).

However, the OT complex has been suggested more recently to be a direct target of Pkc1 action based on three additional observations. First, several OT subunits display two-hybrid interactions with the regulatory domain of Pkc1 (Stt3, Ost1, Wbp1, and Swp1) (255), observations supported by in vitro binding of these subunits to Pkc1. Interestingly, all of these interactions are with domains of the OT subunits predicted to reside in the lumen of the endoplasmic reticulum. It is the C-terminal domain of Stt3, which plays a critical role in substrate peptide recognition and perhaps catalysis, that displays two-hybrid interaction with Pkc1 (153, 358). Second, a *pkc1*Δ mutant (but not *bck1*Δ or *mpk1*Δ mutants) is reduced 50% in OT activity, suggesting that Pkc1 positively regulates OT activity (358). Third, only certain alleles of *STT3* display staurosporine sensitivity, and glycosylation-deficient mutants in most of the other subunits of the OT complex are not staurosporine sensitive (45). Such allele-specific interactions argue against a general deficiency in protein *N*-glycosylation as an explanation for the staurosporine sensitivity of *stt3* mutants.

An alternative interpretation is that Stt3 is a direct target of Pkc1 activity. Perhaps only mutations that partially impair Stt3 function while sparing its ability to be activated by Pkc1 display staurosporine sensitivity. In this model, the OT activity of the weakened form of Stt3 is further reduced by decreased Pkc1 function (i.e., staurosporine treatment).

Chitin synthase 3: the chitin emergency response. The yeast cell wall normally contains approximately 2% chitin. However, a variety of mutations that cause cell wall stress increase chitin levels to as much as 20% of total wall polymers (58, 259, 269). Additionally, the amount of chitin in the cell wall of mating projections is greatly increased (289). In both cases, chitin synthase 3 (Chs3) is responsible for the increased chitin deposition, although the levels of this enzyme do not fluctuate appreciably (48). Under nonstress conditions, most of the Chs3 is maintained in internal stores known as chitosomes within the trans-Golgi network/early endosomal compartments. A recent study revealed that under conditions of cell wall stress (i.e., mild heat shock), Chs3 rapidly exits the trans-Golgi network

and redistributes to the plasma membrane (339). Rapid mobilization of Chs3 to the cell surface was proposed to provide a mechanism for cell wall repair under conditions of wall stress. Conditional mutants in *RHO1* or *PKC1* (but not a *bck1*Δ mutant) were blocked in stress-induced redistribution of Chs3, suggesting that the upper part of the CWI pathway (but not the MAP kinase cascade) was responsible for mounting this response. Chs3 is a phosphoprotein, and Pkc1 function is required for in vivo phosphorylation of Chs3 (339). However, the Chs3 phosphorylation level did not correlate with its stress-induced transport to the plasma membrane. Thus, the key regulatory function of Pkc1 with respect to Chs3 behavior remains unclear.

Another aspect of the chitin response to cell wall damage is the induced expression of *GFA1*. This gene encodes glucosamine-6-phosphate synthase, the first committed and rate-limiting step in the production of UDP-*N*-acetylglucosamine for biosynthesis of chitin (among other products) (243). Under conditions of cell wall stress (e.g., in *gas1* or *fks1* mutants) and in response to treatment with mating pheromone, *GFA1* expression is induced severalfold (36, 316). Ectopic overexpression of *GFA1* is sufficient to drive an increase in chitin deposition (170), indicating that this biosynthetic step is a critical determinant in the amount of chitin produced by Chs3. The *GFA1* gene possesses two Rlm1-binding sites in its promoter, which are responsible for its induction in response to cell wall stress (T. Negishi and D. E. Levin, unpublished). Thus, CWI signaling contributes to the chitin emergency response at least at two levels.

Phospholipid Biosynthesis Targets

CTP synthase is essential for the synthesis of nucleic acids and membrane phospholipids. This enzyme, which in yeast is encoded by the redundant *URA7* and *URA8* genes, converts UTP to CTP, which is required for the production of nucleic acids and all three major membrane phospholipids (i.e., phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine). The *URA7*-encoded enzyme is activated in vitro both by cyclic AMP-dependent protein kinase and Pkc1 (47, 360). In the case of phosphorylation by Pkc1, CTP synthase activity is increased threefold. Although the biological significance of this regulation has not been explored, it may be part of a mechanism to coordinately regulate expansion of the plasma membrane with growth of the cell wall. Similarly, Pkc1 has been shown to phosphorylate the Opi1 protein in vitro (319). Opi1 is a transcriptional repressor of genes encoding phospholipid-synthesizing enzymes. Biochemical analysis revealed that Ser26 is a major site of in vitro Opi1 phosphorylation by Pkc1. However, loss of this phosphorylation site (by mutation to Ala) had only a modest effect on Opi1 phosphorylation in vivo. The S26A form of Opi1 displayed slightly enhanced repression of a reporter gene, prompting the suggestion that Pkc1 functions to alleviate repression of Opi1-regulated genes. However, further tests of this model are required.

Nuclear Functions of Pkc1

Arrest of secretion. Mutants blocked in exocytic protein secretion (i.e., *sec* mutants) reversibly inhibit nuclear import of several nucleoporins and nucleolar proteins, resulting in their relocation to the cytoplasm (233). This so-called arrest-of-secretion response is thought to couple nuclear activities, including transcription of genes encoding ribosomal components, to vesicular transport along the secretory pathway. Additionally, *sec* mutants display a nearly complete transcriptional repression of the genes encoding protein and RNA components of the ribosome as well as tRNAs (185, 218). Similarly, drugs that interfere with different steps of the secretory pathway (e.g., tunicamycin and brefeldin A) also cause repression of ribosomal genes. These effects on ribosome biogenesis are presumed to result from relocalization of nuclear proteins by the arrest-of-secretion response. Because ribosome synthesis accounts for a major portion of the biosynthetic capacity of the cell, it has been suggested that it would be advantageous to the cell to downregulate ribosome biosynthesis in response to slowed membrane expansion (237).

Recent evidence implicates Pkc1 but not the MAP kinase cascade in the arrest-of-secretion response. A null mutant in *PKC1* abrogates the repression of ribosomal protein and rRNA genes caused by the *sec1-1* mutation (237). Similarly, a *pkc1*Δ mutant failed to delocalize the nucleolar protein Fpr3 and the Nsp1 nucleoporin in response to the same late secretory pathway block (232). However, mutations in *MPK1* or *BCK1* fail to block the arrest-of-secretion response.

Nierras and Warner (237) proposed that CWI signaling was being triggered in response to plasma membrane stretch that results from continued protein synthesis (and increased internal osmolarity) in the absence of plasma membrane expansion. This model is supported by several observations. First, inhibition of protein synthesis blocks the arrest-of-secretion response (218). Second, plasma membrane stretch induced by treatment with chlorpromazine induced transcriptional repression of ribosomal protein genes (237). Third, the Wsc cell surface sensors are required for the arrest-of-secretion response, suggesting that the signal emanates from the plasma membrane. Loss of the cell surface sensor Wsc1 in combination with either Wsc2 or Wsc3 blocked repression of ribosomal protein genes by tunicamycin (185), and loss of Wsc2 (or Wsc1 to a lesser extent) diminished Fpr3 and Nsp1 delocalization in a *sec1-1* mutant (232).

In contrast to the model proposed by Nierras and Warner (237), Nanduri and Tartakoff (232) present evidence suggesting that Wsc proteins trapped along the secretory path rather than those at the cell surface may be responsible for signaling the arrest-of-secretion response. No Pkc1 targets involved in the arrest-of-secretion response have been identified to date.

Mitotic recombination. A recessive allele of *PKC1* was isolated in a genetic screen for mutants with an elevated rate of mitotic recombination (128). Although osmotic stabilizers suppressed the growth defect of this mutant at elevated temperature, osmotic support could not suppress the hyperrecombination phenotype, suggesting that the latter was unrelated to cell wall defects. Another allele of *PKC1* not isolated through the mitotic recombination screen was shown to have a similar phenotype, indicating that hyperrecombination is a general defect of *pkc1* mutants. This phenotype was dependent on the recombination protein Rad52. An *mpk1*Δ mutant displayed normal recombination frequencies, suggesting a novel nuclear function for Pkc1 that was not dependent on the MAP kinase

cascade. The relevant target of Pkc1 with respect to recombination has not yet been identified.

G_2/M progression and the mitotic spindle. A *pkc1*Δ mutant is capable of proliferation in the presence of osmotic support, albeit at a somewhat reduced growth rate (177, 253). Although not essential for cell cycle progression, Pkc1 has been suggested to play a role in the passage from G_2 to mitosis. The earliest clue to a cell cycle role for Pkc1 was the finding that Pkc1-depleted cells arrest uniformly with small to medium-sized buds, duplicated DNA, and short nuclear spindles (180). Later, upper CWI pathway components (i.e., Rom2, Rho2, and Mid2) were identified as dosage suppressors of the temperature sensitivity associated with deletion of genes encoding either the kinesin-related protein Kar3 or its associated protein Cik1 (201). Kar3 localizes to the spindle pole bodies and is important for spindle assembly and mitotic chromosome segregation (among other vegetative roles) (200, 248). The function of Cik1 is not clear, but its localization to the mitotic spindle requires Kar3 (248). Similarly, conditional mutants in *SPC110*, which encodes a spindle pole body (SPB) component that binds calmodulin, are suppressed by overexpression of *MID2*, *WSC1*, *ROM2*, or *PKC1* (157, 322). Interestingly, phosphorylation of Spc110 is defective in *pkc1* and *mpk1*Δ mutants (157), suggesting a role for the MAP kinase in SPB function. The recent demonstration that the N-terminal C2-like domain of Pkc1 localizes to the nuclear spindle (64) is similarly supportive of the notion that this kinase has a target at the SPB.

Additional observations support a role for CWI signaling in the function of the microtubule cytoskeleton. A *rom2*Δ mutant and a *pkc1*(Ts) mutant but not an *mpk1*Δ mutant were found to be hypersensitive to the microtubule antagonists benomyl and thiabendazole, respectively (124, 201). Conversely, overexpression of Pkc1 confers thiabendazole resistance on wild-type cells (124). Interestingly, these authors also demonstrated that a *pkc1*(Ts) mutant displays a G_2 delay and aberrant nuclear positioning in a fraction of the cells at semipermissive temperature, supporting the notion that Pkc1 plays a role in microtubule or SPB function.

Several recent studies have revealed genetic connections between Pkc1 and the ATP-dependent chromatin remodeler complex called the RSC (for Remodels the Structure of Chromatin) (43, 124, 282). This complex serves an essential but poorly understood function in progression from G_2 to mitosis. Conditional mutants in the catalytic subunit of the RSC complex encoded by *NPS1*/*STH1* (335) display growth arrest at G_2/M under restrictive conditions, increased sensitivity to thiabendazole and benomyl, a decrease in chromosome transmission fidelity, and defects in actin cytoskeletal organization (43, 124). Overexpression of several upstream components of the CWI pathway, including Pkc1 (but not components of the MAP kinase cascade), suppressed all of these phenotypes (43, 124). Moreover, temperature-sensitive mutations in *PKC1* are synthetically lethal with mutations in various RSC subunits (124, 282). The observation that RSC mutants do not display cell lysis defects (124, 282) suggests that the role of Pkc1 in this context is unrelated to the cell wall. Because Pkc1 does not phosphorylate RSC in vitro, it has been proposed that Pkc1 and RSC contribute in parallel to a common function in the G_2/M transition (282).

A microtubule-related function for the RSC complex is suggested by its genetic interactions with *BIM1*, which encodes a homolog of human microtubule-binding protein EB1 (299, 329, 352). Bim1 binds along the microtubule network but is concentrated at the distal tip. Bim1 together with Kar9 constitute a cortical microtubule capture site (27). The *BIM1* gene was isolated through a dosage suppressor screen of *nps1*(Ts) and further shown to suppress the thiabendazole sensitivity of this mutant, similar to *PKC1* overexpression (124). The G_2 delay and nuclear position defect of a *pkc1*(Ts) mutant but not its cell lysis defect were also suppressed by overexpression of *BIM1*. However, overexpression of Pkc1 did not alleviate the thiabendazole sensitivity of a *bim1*Δ mutant, prompting the suggestion that Bim1 functions downstream of Pkc1 (124). Like the connection between Pkc1 and RSC, the connection between Pkc1 and Bim1 was proposed to be indirect. However, new evidence that seems to place a pool of Pkc1 at the nuclear spindle (64) suggests that one or more spindle or SPB components are substrates for Pkc1. A recent study also implicated RCS in the establishment of sister chromatid cohesion (19).

SPB duplication. As noted above, the growth defects of conditional mutants in several SPB components (e.g., *kar3*, *cik1*, and *spc110*) are suppressed by overexpression of upper components of the CWI signaling pathway (157, 200, 322). The MAP kinase branch of this pathway has also been implicated in SPB duplication (157). An early step in SPB duplication requires Cdc31 and Kar1, two SPB components, and Dsk2 and Rad23, a pair of ubiquitin-like proteins. Overexpression of *WCS1* or *PKC1* suppresses the temperature-sensitive growth arrest of conditional mutants in *CDC31* or *KAR1* or a double *dsk2*Δ *rad23*Δ mutant. Interestingly, overexpressed Mpk1 and constitutively active *BCK1* (*BCK1-20*) also appear to be weak suppressors of the conditional *kar1* mutant. Whether the involvement of CWI signaling in SPB duplication suggested by this study is related to the role of Pkc1 in the G_2/M transition discussed above is not clear. Certainly, the nuclear functions of Pkc1 and the Mpk1 MAP kinase are ripe for further investigation.

Why should a signaling pathway whose principal function is to regulate cell wall biogenesis also serve functions related to entry into mitosis? The CWI signaling pathway may coordinate cell surface expansion with the mitotic cell cycle. Although clearly not required for progression of the cell cycle, the periodic fluctuation of CWI signaling during bud formation may provide temporal cues to the mitotic cycle. In such a model, activation of Pkc1 in response to the cell wall stress associated with budding (during G_1/S) could augment nuclear microtubule functions in preparation for mitosis.

Pkc1 does not control depolarization of the actin cytoskeleton. Delley and Hall (63) observed that cell wall stress causes a transient depolarization of GS (Fks1 and Rho1) along with the actin cytoskeleton. It was proposed that delocalization of the actin cytoskeleton is a mechanism to repair cell wall damage. These authors further proposed actin depolarization to be mediated by a branch of the CWI pathway that includes Rho1 and Pkc1 but not the MAP kinase cascade. According to their model, the CWI pathway would drive repolarization of actin through the MAP kinase cascade only after the wall damage had been repaired. Thus, different branches of the same pathway would mediate actin depolarization and repolarization in a sequential manner.

The conclusions of Delley and Hall (63) were based on two

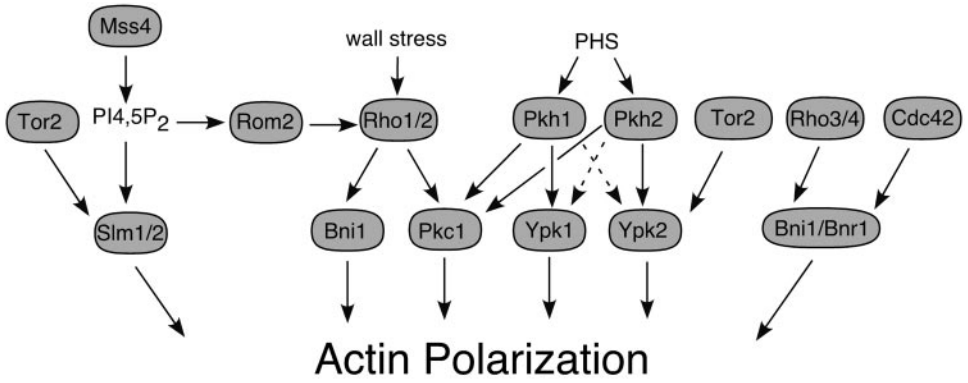

FIG. 6. Several signaling pathways converge to drive actin polarization. Rho1 and -2, the regulators of the CWI signaling pathway, influence actin through the Bni1 (and probably Bnr1) formin protein as well as through the Pkc1-activated MAP kinase cascade. Rho3 and -4 and Cdc42 drive actin polarization through Bni1 and Bnr1. The Pkh1/2 protein kinases are activated by phytosphingosine (PHS) and contribute to actin polarization in at least two ways—activation of Pkc1 and of Ypk1/2. Ypk2 (and presumably Ypk1) also require phosphorylation by Tor2 to be active. The action of the PI4P 5-kinase Mss4 at the plasma membrane generates $PI4,5P_2$, which recruits both the Rho1 GEF Rom2 and the Slm1/2 proteins to the plasma membrane through their PH domains. Slm1 and -2 must also be phosphorylated by Tor2 plasma membrane association. Their role in actin polarization is not understood, but they may represent a node for the integration of signals from Mss4 and Tor2.

sets of observations. First, *wsc1*Δ and *rom2*Δ mutants but not *bck1*Δ or *mpk1*Δ mutants were partially deficient in the depolarization of their actin cytoskeleton in response to mild heat shock. Second, growth-inhibitory levels of overexpressed constitutive Rho1 or Pkc1 but not Bck1 or Mkk1 induced actin depolarization. Importantly, these authors did not report examination of a *pkc1*Δ mutant, which depolarizes its actin cytoskeleton normally in response to mild heat shock (D. E. Levin and B. Philip, unpublished), thus excluding Pkc1 as a factor. The observation that Pkc1 is not required for heat shock-induced actin depolarization calls into question the significance of the observed depolarization induced by lethal levels of Pkc1 and Rho1 (63).

Another interpretation of Delley and Hall's results takes into account the fact that *wsc1*Δ and *rom2*Δ mutants are only partially blocked in CWI signaling due to functional redundancies. These mutants live in a chronic state of wall stress. Because CWI signaling drives actin polarization, chronic wall stress causes preadaptation to additional stress that would normally result in actin depolarization. Chronic exposure to a low dose of calcofluor white also blocks heat shock-induced actin depolarization (D. E. Levin and B. Philip, unpublished). Because mutants that are completely blocked for signal transmission (e.g., *pkc1*Δ, *bck1*Δ, or *mpk1*Δ) cannot adapt to wall stress, they display no resistance to actin-depolarizing stresses. Put another way, constitutive activation of actin polarization signaling in *wsc1*Δ or *rom2*Δ cells (through both Mpk1 and Bni1/Bnr1) makes these mutants appear to be defective in actin depolarization. The observation that deletion of *MPK1* largely restores heat shock-induced actin depolarization to *wsc1*Δ cells (D. E. Levin and B. Philip, unpublished) supports this interpretation. Thus, although the CWI pathway clearly drives polarization of the actin cytoskeleton, the available evidence does not support a role for this pathway in actin depolarization.

INTERFACE WITH OTHER SIGNALING PATHWAYS

Several pathways that all appear to converge to regulate polarization of the actin cytoskeleton intersect directly or indirectly with CWI signaling. Those for which credible connections have been established include phosphoinositide metabolism at the plasma membrane, a protein kinase cascade involving the Pkh1/2 and Ypk1/2 protein kinase pairs, and the Tor protein kinases. The proposed relationship of these pathways to the CWI signaling pathway and to actin polarization is presented in Fig. 6.

Pkh1/2 and Ypk1/2

The yeast Pkh1 and Pkh2 genes encode functional counterparts of the mammalian 3-phosphoinositide-dependent protein kinase 1 (PDK1) (42, 134) that have been implicated in CWI signaling. Upon activation by 3-phosphoinositides (331) or sphingosine (158), mammalian PDK1 phosphorylates sites within the activation loops of a variety of protein kinases, including cyclic AMP- and cyclic GMP-dependent protein kinases, Akt/PKB, several PKCs and PKC-related kinase isoforms, p70 S6 kinase, p90 RSK, and serum- and glucocorticoid-inducible kinase (SGK) isoforms. Collective evidence suggests that phosphorylation at the so-called PDK1 site is required for full activation of these target kinases. By contrast to PDK1, its yeast homologs are not regulated by phosphoinositides (42) but are modestly stimulated in vitro by nanomolar concentrations of phytosphingosine, the major sphingoid base in yeast (92, 370).

Pkh1 and -2 serve an essential but overlapping function in the maintenance of cell wall integrity (134) (Fig. 6). A *pkh1*(Ts) *pkh2*Δ mutant exhibits both actin polarization and osmoremedial cell lysis defects at the restrictive temperature. Growth is partially restored by constitutive activation of Pkc1, Bck1, or Mkk1, suggesting a connection between Pkh1/2 function and CWI pathway signaling. Additionally, Pkh1 and Pkh2 phosphorylate Pkc1 in vitro at T983 (92, 134), the activation loop residue analogous to those phosphorylated in the targets of mammalian PDK1. Pkh1/2 function is required for full activation of Pkc1 in response to mild heat shock, and elimination of the Pkh1/2 phosphorylation site in a *pkc1-T983A* mutant results in failure to complement a *pkc1*(Ts) allele.

In addition to activating Pkc1, Pkh1 and -2 phosphorylate and activate a pair of functionally overlapping protein kinases, Ypk1 and Ypk2 (42). The *YPK1/2* genes encode an essential pair (46) of functional analogs of mammalian SGK, which have also been implicated in CWI signaling (42, 280, 291). Pkh1 appears to target Ypk1 preferentially (at T504), whereas Pkh2 prefers to phosphorylate Ypk2 (at T501) (42, 280). Consistent with the notion that Ypk1 and -2 are at least indirect targets of phytosphingosine signaling, overexpression of Ypk1 confers resistance to the growth-inhibitory sphingosine analog myriocin (also known as ISP-1), which causes sphingolipid depletion (324).

A *ypk1*Δ mutant grows slowly and displays an actin polarization defect (46, 280, 291). Additionally, a *ypk1*(Ts) *ypk2*Δ [*ypk*(Ts)] strain exhibits osmoremedial cell lysis at the restrictive temperature. A comprehensive suppressor analysis of this mutant's growth defect of was presented recently (280). The *ypk*(Ts) strain could be suppressed by overexpression of *PKC1* or mutational activation of *BCK1* (*BCK1-20*), suggesting a connection to the CWI pathway (280). However, a *ypk1*Δ *ypk2*Δ mutant is neither osmoremedial nor suppressed by *PKC1* or *BCK1-20* (46, 280) indicating that some low level of Ypk activity is required for suppression.

Significantly, among the variety of recessive mutational suppressors of the *ypk*(Ts) growth defect reported were many genes involved in the maturation of the Asn-linked core oligosaccharide, which is added en bloc to cell wall mannoproteins (280). This unexpected finding suggested that additional cell wall stress somehow helps the *ypk*(Ts) mutant. Consistently, two dosage suppressors of *ypk*(Ts) identified in the same study were *EXG1*, which specifies an exoglucanase, and *CTS2*, which encodes a chitinase. These collective suppression data led the authors to suggest that the mechanism by which wall defects suppressed the *ypk*(Ts) mutant was by triggering activation of Rho1/Pkc1 signaling and that the Ypk1/2 kinases signal through a novel pathway for CWI that is parallel to the Rho1/Pkc1-dependent pathway.

The cellular targets of Ypk1/2 have yet to be identified. However, a *ypk1*Δ mutant has been reported to display reduced ability to activate Mpk1 in response to mild heat shock (291), prompting the suggestion that Ypk1 and -2 contribute in an unspecified way to the activation of Mpk1. Nevertheless, taking into consideration the cell wall stress-mediated suppression of the *ypk*(Ts) growth defect observed by Roelants et al. (280), it seems more likely that the two pathways act in parallel. Suppression of the *ypk*(Ts) growth defect by additional wall stress would seem unlikely if Pkc1/Mpk1 signaling is actually compromised in this mutant.

What is the significance of sphingolipid regulation of Pkh1/2? Membrane microdomains enriched in sphingolipids and sterols, known as lipid rafts, are important for the delivery of certain proteins to the cell surface of yeast (20). In this regard, it is interesting that Pkh1 and -2 localize mainly to cortical puncta that are distinct from actin patches, which may be lipid rafts (280). An attractive hypothesis is that plasma membrane growth by fusion of sphingolipid-enriched vesicles is monitored and coupled to cell wall expansion through Pkh1/2 regulation of Ypk1/2 and Pkc1 (280). Another possibility concerns the requirement of sphingolipid signaling to Pkh1/2 for the internalization step of endocytosis (92). Both an *lcb1* mutant, deficient in sphingolipid biosynthesis, and a *pkh1/2 ypk*(Ts) mutant are defective in endocytosis (92). Moreover, the endocytic defect of *lcb1* is suppressed by overexpression of Pkh1/2 or Pkc1 (92, 93). Ypk1 has also been implicated in the internalization step of endocytosis (62). However, proper actin assembly is essential for endocytosis (303), and mutants in any of these protein kinases display abnormal actin polarization, suggesting that their endocytic defects may be indirect.

Tor Regulation of the Actin Cytoskeleton

Tor1 and Tor2 (*T*arget *o*f *r*apamycin) (115) are structurally similar members of a family of phosphatidylinositol kinase-related protein kinases. Despite their sequence similarity to lipid kinases (168), they have been shown to possess Ser/Thr-specific protein kinase activity (2, 290). They were identified initially by dominant mutations that conferred resistance to the antibiotic rapamycin. The *TOR2* gene is essential, but cells lacking *TOR1* exhibit only mild growth defects. *TOR2* has been proposed to have two essential functions, based on two sets of observations. First, cells lacking *TOR2* exhibit different growth arrest phenotypes than cells lacking both *TOR1* and *TOR2*. Specifically, cells lacking either *TOR1* and *TOR2* or treated with rapamycin arrest uniformly in the G_1 phase of the cell cycle (22). This growth arrest results from a block in the initiation of protein synthesis. By contrast, cell lacking only *TOR2* arrest randomly in the cell cycle (39, 118, 168). Second, overexpression of *TOR1* cannot suppress loss of *TOR2*.

Based on these observations, Tor2 has been suggested to share an essential function with Tor1 in the control of G_1 progression and to possess a second essential function that is independent of Tor1 (293). The molecular basis of these genetic results became clear with the recent discovery that Tor1 and Tor2 can participate in two distinct complexes, TORC1 and TORC2 (190). TORC1 is rapamycin sensitive, contains either Tor1 or Tor2, and mediates the shared Tor function. TORC2 is rapamycin insensitive, contains Tor2 (but not Tor1) and five additional subunits (Avo1, Avo2, Avo3, Bit61, and Lst8) (190, 271), and mediates the Tor2-unique function.

The Tor2-unique function is important for organization of the actin cytoskeleton (293). This conclusion was based on the dual observations that a *tor2*(Ts) mutant displays a randomized actin distribution at the restrictive temperature and that the growth defect of this mutant is suppressed by overexpression of Tcp20, an actin-specific chaperone. The actin organization defect was connected genetically to Rho1 signaling by the finding that the *tor2*(Ts) growth arrest and actin depolarization defects were suppressed by overexpressing CWI pathway components at any level (116, 117, 292). Additional support for this connection came from the isolation of mutational suppressors of *tor2*(Ts) that activate CWI signaling by causing cell wall damage (e.g., *fks1* and *gas1*) (26). Indeed, destabilization of the cell wall by a low concentration of sodium dodecyl sulfate in the growth medium also suppressed the *tor2*(Ts) growth defect. These observations are reminiscent of the suppression of *ypk*(Ts) mutants by mutations that cause cell wall damage.

The additional finding that Rom2 exchange activity was reduced in a *tor2*(Ts) mutant (292) provided the only biochemical connection to date between Tor and CWI signaling and prompted the authors to propose that Tor2 signals to the actin

cytoskeleton through Rom2 and the CWI pathway. However, this experiment was conducted with extracts from cells that had been growth arrested at the restrictive temperature for 8 h, leaving open the possibility that the reduction in Rom2 activity reflected the growth state of the cells. Aside from this single observation, all of the genetic results are equally consistent with a model in which Tor2 signals to the actin cytoskeleton through a pathway that is parallel to CWI signaling. Supporting the notion of parallel pathways is the finding that thermal activation of Mpk1 is not impaired in a *tor2*(Ts) mutant (117; D. E. Levin, unpublished), indicating that at least stress-induced CWI signaling is not dependent on Tor2 function.

One clue to the role of Tor2 in actin polarization comes from the recent identification of a pair of essential plasma membrane-associated PH domain proteins, Slm1/2, in a synthetic lethal screen with an *mss4 tor2*(Ts) mutant (14). As mentioned above, like Mss4, Slm1 and Slm2 are important for actin polarization. High-throughput two-hybrid analyses suggested that Slm1 and Slm2 interact with the Avo2 subunit of the TORC2 complex (138, 336). Coprecipitation experiments confirmed this for Slm1 (14). Moreover, in addition to the Mss4-generated $PI4,5P_2$ required for the association of Slm1/2 with the plasma membrane, proper localization of these proteins is also dependent on phosphorylation by Tor2. Slm1 was transiently dephosphorylated in response to mild heat shock with similar kinetics to the depolarization/repolarization of the actin cytoskeleton observed in response to heat stress. Therefore, Slm1/2 may represent a regulatory node that integrates signals from Tor2 and the Stt4/Mss4 pathway to drive actin polarization (Fig. 6).

Though both Tor2 and Ypk1/2 protein kinases may function along parallel pathways to CWI signaling, the similarities in their mutant behaviors suggest that these protein kinases may function in a common pathway. Both the Tor2 and Ypk1/2 kinases are required for actin polarization. Moreover, both *tor2*(Ts) and *ypk*(Ts) mutants are suppressed by cell wall stress or by overexpression or mutational activation of CWI signaling. Additional support for a model in which these kinases function in a common pathway is the observation that *Schizosaccharomyces pombe* Tor1 phosphorylates and activates the Ypk ortholog Gad8. Finally, a recent report revealed that a constitutively active form of Ypk2 suppresses the growth defects of both a *tor2*(Ts) strain and a *tor2*Δ mutant (147). These authors also demonstrated that Tor2 phosphorylates Ypk2 at a pair of residues near its C terminus (S641 and T659) and that these phosphorylations are required for Ypk2 protein kinase activity. These findings provide an additional mechanism by which Tor2 contributes to actin polarization (Fig. 6).

PERSPECTIVES AND FUTURE DIRECTIONS

Early views of the yeast cell wall as a rigid and static structure of limited interest have given way in the past decade to a new recognition of its highly dynamic nature. The discovery of multiple signaling pathways involved in regulating the reorganization of the cell wall in response to various environmental cues has led to a new understanding of the interrelated nature of the actin cytoskeleton, polarized secretion, and cell wall remodeling. However, many of the molecular details remain to be discovered, particularly as concerns the response of the cell polarization machinery to signaling from the cell surface.

Several important questions concerning CWI signaling have presented themselves as a result of recent developments. First, what is the mechanism by which the cell surface sensors detect cell wall stress? Are they mechanosensors that respond to contact between their stiff periplasmic ectodomains and the cell wall? This would certainly provide a simple mechanism for detecting outward membrane stretch. Alternatively, they may engage in specific molecular interactions with cell wall components, as suggested by the Cys-rich motifs of Wsc proteins. In either case, the mechanism by which they communicate with the intracellular signaling apparatus remains largely shrouded. For example, is a signal transmitted through the transmembrane region to induce a conformational change in the cytoplasmic domain, or do these proteins merely provide an aggregation site for Rho1 and its regulators? A third possibility is that these sensors, like the Sln1 osmosensor, detect turgor pressure in the plasma membrane independently of the cell wall. This seems less likely considering the importance of the mannosylated ectodomain to their function.

A second important issue centers on the identification of nontranscriptional targets of CWI signaling. Specifically, how does Pkc1 exert its effects on the G_2/M transition and other nuclear events? Numerous lines of evidence implicate Pkc1 in a diverse array of nuclear functions, from localization of nucleolar and nuclear pore proteins to mitotic recombination to SPB duplication. The recent finding of a pool of nuclear Pkc1, some of which is associated with the nuclear spindle, suggests that one or more microtubule or SPB proteins might be direct targets of this kinase. A related question concerns the mechanism by which Mpk1 drives polarization of the actin cytoskeleton and how this regulation interfaces with other signaling pathways that also impact actin organization. Identification of additional substrates not only for Mpk1 but also for the Ypk1/2 kinases will undoubtedly offer new insight into this problem.

A third question concerns the role of CWI signaling in the oxidative stress response. It is not immediately obvious why a pathway that appears to be designed to detect events at the cell surface would be important for responding to an intracellular stressor such as hydrogen peroxide. Perhaps a secondary signaling pathway that involves some components of the CWI pathway but not the cell surface sensors or Mpk1 exists for this purpose. There are well-established precedents for shared use of MAP kinase cascade components among separate signaling pathways (reviewed in reference 121).

A final question concerns the nature of signal integration and output by Rho1. This issue is not only important for better understanding CWI signaling in yeast but is of general interest for understanding complex signaling pathways in higher eukaryotes. It is clear that the Wsc/Mid family members are not entirely functionally redundant, yet it appears that most, perhaps all, signal to Rho1. It seems likely that differences in the source or intensity of wall stress signals trigger different combinations of sensors, which is interpreted differently by the Rho1 switch. Evidence is beginning to emerge to support the notion of different sensors activating different pools of Rho1. To address this question fully, better tools will be required to monitor the various functions of this diverse G-protein.

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